Mechanisms and novel therapies in cervical spinal cord injury

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Abstract

Recent epidemiological data indicate that more than half of SCI patients have injuries of the cervical spine. There is no satisfactory treatment for these injuries either in the acute or the chronic phase. Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid that is essential in brain development and has structural and signalling roles. Acute DHA administration has been shown to improve neurological functional recovery following injury in rodent thoracic spinal cord injury (SCI) animal models.

In this thesis, we characterized a cervical SCI model comprising a hemisection lesion applied at the C4-5 level of the rat spinal cord, and tested the effects of an acute treatment with 250 nmol/kg DHA delivered intravenously 30 minutes after injury. The acute intravenous bolus of DHA not only increased the number of neuronal cells spared at three weeks following injury but also resulted in robust sprouting of uninjured corticospinal and serotonergic fibres. Next, we used a mouse pyramidotomy model to confirm that this robust sprouting was not species or injury model specific. We demonstrated that the number of V2a interneurons contacted by collateral corticospinal sprouting fibres is positively correlated with skilled motor recovery. To address the mechanism behind the neuroplasticity-promoting effect of DHA, we investigated the expression of miR-21 and phosphatase and tensin homolog (PTEN) in cortical neurons and raphe nuclei after DHA treatment. We found that DHA significantly up-regulates miR-21 and down-regulates PTEN in corticospinal neurons one day after SCI. Down-regulation of PTEN by DHA was also seen in dorsal root ganglion (DRG) neuron

cultures and was accompanied by increased neurite outgrowth. Lastly, we investigated whether DHA treatment combined with specific-task rehabilitation maximized the recovery of skilled forelimb function following cervical SCI. The rats receiving combined therapy achieved greater skilled forelimb functional recovery compared to DHA treatment or rehabilitation only.

In summary, this study shows that DHA has therapeutic potential in cervical SCI and provides evidence that DHA could exert its beneficial effects in SCI via enhancement of neuroplasticity.

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List of Abbreviations

5-HT	5-hydroxytryptamine
AA	Arachidonic acid
ALA	Alpha-linolenic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	Adenomatous polyposis coli
ASIA	American Spinal Injury Association
Atf-3	Activating transcription factor 3
BBB	Basso, Beattie, Bresnahan
BDNF	Brain derived neurotrophic factor
BMSC	Bone marrow-derived stromal cells
CaMKII	Ca2+/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
ChABC	Chondroitinase ABC
CNS	Central nervous system
cPLA2	Cytosolic phospholipase A2
CREB	cAMP response element-binding protein
CSPG	Chondroitin sulphate proteoglycans
CST	Corticospinal tract
DHA	Docosahexaenoic acid
DRG	Dorsal root ganglion
EPA	Eicosapentaenoic acid

FABP	Fatty-acid-transport proteins
FLS	Forelimb locomotor scale
GABA	Gamma-aminobutyric acid
Gap-43	Growth associated protein – 43
GluR1	Glutamate receptor 1
HSP-27	Heat shock protein 27
Iba-1	Ionized calcium-binding adapter molecule-1
IGF-1	Insulin-like growth factor
JAK	Janus-activated kinase
LT	Leukotrienes
miRNA	MicroRNA
mTOR	Mammalian target of rapamycin
NeuN	Neuron specific nuclear protein
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NR2B	NMDA receptor subunit 2B
NT3	Neurotrophin-3
OEG	Olfactory ensheathing glia
PG	Prostaglandin
PI3K	Phosphatidylinositol 3-kinase
PIPs	Phosphatidylinositol phosphates
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PPAR	Peroxisome proliferator activated receptors

PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acid
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCI	Spinal cord injury
SOCS-3	Suppressor of cytokine signaling 3
SSC	Saline-sodium citrate
STAT	Signal Transducer and Activator of Transcription
STAT3	Signal transducer and activator of transcription – 3
TBI	Traumatic brain injury
TRAAK	TWIK-related arachidonic acid stimulated potassium channel
TREK-1	Twik-related potassium channel 1

Authors Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Barts and the London School of Medicine and Dentistry, Queen Mary University of London. The research described within this thesis was performed in the Centre for Neuroscience and Trauma within the Blizard Institute of Cell and Molecular Science. The research was carried out under the supervision of Professor Adina T. Michael-Titus, Professor John V. Priestley and Dr. Ping K. Yip and is my own work unless stated otherwise.

Posters and publication arising from this work

Poster Presentations

1. Conference: Meeting of British Neuroscience Association (BNA): Festival of Neuroscience ,The Barbican Centre, London. 2013
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Authors: Zhuo-Hao Liu, Ping. K. Yip, Adina T. Michael-Titus, John V. Priestley

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Title: Effects of ω3-Polyunsaturated Fatty Acids in a Rodent Model of Cervical Spinal Cord Injury
Authors: Zhuo-Hao Liu, Ping. K. Yip, Adina T. Michael-Titus, John V. Priestley

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Publication

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1 General Introduction

Spinal cord injury (SCI) is a devastating and debilitating condition that affects the ability of individuals to carry out normal activities. According to World Health Organization statistics, it is estimated that the global annual incidence of SCI is 40 to 80 cases per million population. Most SCI patients are young adults (Cripps et al. 2011). In addition to the poor life quality associated with paralysis, sensory loss, intractable pain, pressure sores, and urinary tract infections, people with SCI are 2 to 5 times more likely to die prematurely than people without SCI (http://www.who.int/mediacentre/factsheets/fs384/en/).

There is currently no effective treatment for SCI. Current treatments in the acute stage, such as acute decompression (Fehlings et al. 2006; Fehlings et al. 2012) or administration of methylprednisolone (Hurlbert et al. 2008) are controversial or have limited efficacy. In the late stage, neuroplasticity within the central nervous system (CNS) following rehabilitative training is one of the most successful strategies clinically (Wang et al. 2011; Fouad et al. 2012).

The phospholipids in CNS membranes contain several different fatty acids. One compound, the omega-3 polyunsaturated fatty acid DHA, accounts for approximately 50% of the polyunsaturated fatty acid (PUFA) in CNS membranes and plays an essential role in brain development and plasticity. Omega-3 PUFAs have been shown to have therapeutic potential in a variety of neurological conditions (Hashimoto et al. 2002;

Wu et al. 2004; Michael-Titus 2007; Dyall et al. 2008).

Before I was involved in these doctoral studies, our laboratory had demonstrated that DHA improved locomotor function following compression or hemisection injury in rodent animal models of thoracic SCI (King et al. 2006; Huang et al. 2007). The aim of this thesis is to study the effects of DHA after an injury of the spinal cord at cervical level. We will characterize the neuroprotective effect of DHA and also the potential of this treatment to enhance neuroplasticity after injury, with or without rehabilitative training.

1.1 Cervical SCI

SCI is a very disabling condition that has a profound impact on the patients' lives. Epidemiological data show that 51% of SCI patients have injuries in the cervical spine, with the most common neurological level being C5, followed by C4 and C6 (Sekhon et al. 2001; Dunham et al. 2010). This type of injury can cause severe impairments, including impaired use of the upper and lower extremities, loss of physical sensation, respiratory problems, bowel, bladder, and sexual dysfunction. Regaining partial or full function of the arm and/or hand could lead to significant improvements in the patient's quality of life, and is considered to be a priority for patients suffering cervical cord injuries (Anderson 2004).

Neurorecovery in the upper extremities is of paramount importance for the degree of functional independence. Patients with high level injuries (i.e. C1-4) require complete assistance with activities of daily living, bed mobility and transfers. Sometimes, these patients also require permanent mechanical ventilation and suctioning to clear secretions (Lanig et al. 2000). Patients with C5 injuries can perform active elbow flexion and can therefore perform some simple tasks. These patients typically require 10 hours a day of personal care. Patients with C6 injuries have active wrist extension, passive finger flexion, and opposition of the second digit and the thumb. Shoulder stability is supported because they retain full innervation of the rotator cuff. Grip strength can be improved using orthosis. The patients typically require 6 hours a day of personal care (Medicine 2000). Patients with C7 injuries have retained strength of the triceps. Some assistance may be required for toileting and dressing activities, particularly for the lower

extremities. However, eating, grooming, and dressing of the upper extremities can be performed independently (Medicine 2000) .

1.1.1 Motor deficits following cervical SCI

After SCI, one of the most devastating consequences is the loss of motor system control. In humans, the American Spinal Injury Association (ASIA) Impairment Scale is widely used to determine the level of SCI and the extent and severity of a patient's SCI and help determine future rehabilitation and recovery needs. The assessment is ideally completed within 72 hours after the initial injury. The patient's grade is based on how much sensation he or she can feel at multiple points on the body, as well as their performance in tests of motor function. Clinically, an improvement in ASIA score is the main outcome measure for the efficacy of treatments and ranges from A to E, where A is a complete lack of motor and sensory function below the level of injury (including the anal area) and E is normal motor and sensory function. In rodent experimental models, the main functional measurement that is taken in thoracic SCI is an assessment of locomotor recovery, whereas in cervical SCI the evaluation of skilled forelimb control is used as an endpoint.

All movement produced by the skeletal musculature is dependent on lower motor neurons in the ventral horn spinal cord gray matter. The lower motor neurons form a pool that innervates a single muscle and their axons extend up and down for several spinal cord segments. Ventral horns contain motor neurons of various type (α-motoneurons reaching skeletal muscle fibres, and γ-motoneurons innervating intra-fusal motor fibres in muscle spindles). The ventral horns are larger at the levels of the cervical

and lumbar enlargements because of motor neurons innervating limb muscles. After SCI, there is damage to lower motor neurons in the spinal cord, which results in paralysis of the affected muscles and loss of reflexes and muscle tone.

1.1.1.1 Hindlimb: locomotion function

After SCI, all animals suffer locomotor deficits, including human beings. However, there are differences in the degree of recovery between rodents and primates. Considerable recovery of locomotor function is generally achieved when the ventral and lateral white matter is preserved (Basso et al. 2002). Experimental studies have led to the discovery that the basic rhythmic patterns of each limb involved in locomotion are dependent on "central pattern generators"- a local circuit in the spinal cord responsible for the alternation of flexion and extension of limbs. Following transection of the thoracic spinal cord, a cat or rat's hindlimb still makes coordinated locomotor movements if the animal is supported and placed on a moving treadmill (Grillner et al. 1979; Edgerton et al. 2002). However, in non-human primates or humans, the ability to execute rhythmic stepping is permanently impaired after SCI (Courtine et al. 2005). Another possible reason for the lack of stepping is that the corticospinal tract (CST) projections to the lumbosacral enlargement are less than to the cervical cord in rodents (Muir et al. 1999). Injury to the dorsal CST does not produce persistent and severe locomotion deficits, except deprivation of fine paw and digit movements (Muir et al. 1999; Metz et al. 2000). In addition, it seems that the occurrence of bipedal locomotion relies greatly on upper motor neuron pathways, such as those involved in postural control (Rossignol et al. 2011).

1.1.1.2 Forelimb: skilled movement

More than half of the people with SCI have injuries involving the cervical spinal cord (National Spinal Cord Injury Statistical Center, 2014), and neurological impairment in those patients involves not only the ability of lower limb movement but also upper limb function (e.g. skilled upper limb use). A majority of quadriplegic patients have agreed that regaining arm and hand function, not locomotor ability or trunk ability, is the highest priority for improving life quality (Anderson 2004).

Skilled forelimb reaching is a sensorimotor ability shared by many species of animals including humans (Iwaniuk et al. 2000). Skilled reaching requires both sensory information and motor input, which involves a variety of neuronal structures including the brain cortex (Martinez et al. 2009), the CST (Li et al. 1997; Starkey et al. 2005), the red nucleus (Whishaw et al. 1998), cerebellum (Garwicz 2000), reticular formation (Davidson et al. 2006; Riddle et al. 2009), and the basal ganglia (Whishaw et al. 1986; Miklyaeva et al. 1994) (Fig 1.1). Recently, skilled forelimb reaching has been examined in rats with unilateral spinal cord hemisection. After cervical SCI, the rats make fewer attempts to retrieve food pellets and are less successful at grasping the food pellets when compared to sham-operated animals (Anderson et al. 2005). Skilled forelimb task evaluation is useful to determine the effect of a particular therapy in rat cervical SCI models.

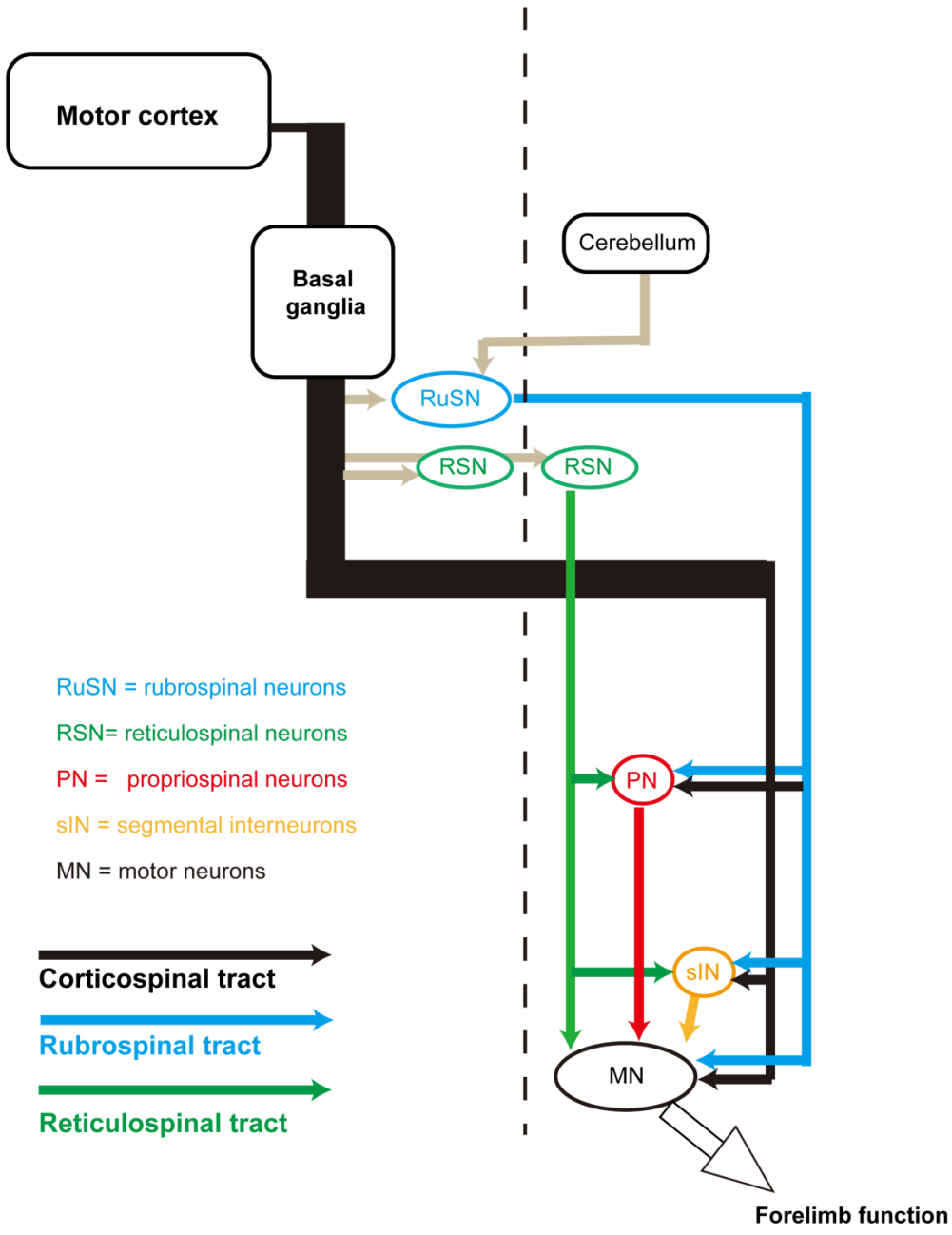

Figure 1-1 Pathways from the sensorimotor cortex to hand motoneurons
The direct corticomotoneuronal pathway is primarily originated from the primary motor cortex. There are several indirect routes, which could mediate the cortical command to hand and arm motoneurons located in cervical segments, such rubrospinal tract and reticulospinal tract. In addition, the propriospinal neurons (PNs) and segmental interneurons (sINs) could relay the cortical inputs to motoneurons. Adapted from (Riddle et al. 2009).

1.1.2 Cervical spinal cord hemisection in human beings

High cervical unilateral hemisection injuries in humans result in the Brown-Séquard syndrome (Brown-Séquard 1868), in which one-half of the spinal cord is completely injured. The classical clinical presentation is ipsilateral motor paralysis from the injury level due to damage to the descending CST. Remarkably, the limbs on the contralateral side are insensitive to pain and temperature due to interruption in the crossed spinothalamic tract, while the limbs on the ipsilateral side retain their perception of pain and temperature (Herr et al. 1987). Interestingly, patients with Brown-Séquard syndrome are known to have good recovery of leg control and locomotor function (Little et al. 1985; Roth et al. 1991). On the contrary, arm and hand functions have limited recovery. They remain severely impaired or fully paralyzed in these patients (Levi et al. 1996).

1.1.3 Cervical spinal cord hemisection in rats

Numerous studies have investigated motor function after cervical spinal cord hemisection in rats (Webb et al. 2002; Anderson et al. 2005; Martinez et al. 2009; Martinez et al. 2010; Filli et al. 2011). In rats, the ispilateral forelimb and both hindlimbs are severely impaired following cervical hemisection at the acute stage. After 2-4 weeks, the rats display no impairment of locomotor function of the hindlimbs (Webb et al. 2002; Filli et al. 2011). In contrast, the ipsilateral forelimb remains rigid or flaccid, with constant dragging. These findings parallel the clinical observations in patients with Brown-Séquard syndrome, who present good recovery of leg function, but poor performance of arm and hand function (Levi et al. 1996). The similarity in lesion outcome across

different species with cervical hemisection could be explained by unilateral cervical hemisection leading to a full unilateral ablation of commands from each supraspinal pathway, independent of its specific function. Given the similar anatomy of most descending tracts (Lemon 2008), this results in a similar neurological deficit between species.

Skilled forelimb reaching has been examined in rats with cervical hemisection. Rats with this type of injury make fewer attempts to reach for food pellets and are less successful at retrieving food pellets when compared to sham operated animals (Anderson et al. 2005). The impairment of skilled forelimb reaching results from unilateral damage to the ascending (dorsal column-medial lemniscal and post-synaptic dorsal column) pathways or the descending component (main crossed corticospinal tract) of the cervical spinal cord dorsal column pathway (Webb et al. 2005). The ascending component of the dorsal columns was investigated with respect to its importance in performing skilled movement in rats (McKenna et al. 1999). The data showed that rats recovered food retrieval ability by making adjustments in the whole body and forelimb movement when retrieving a food pellet (McKenna et al. 1999). However, following unilateral injury to the main dorsal corticospinal tract (descending pathway), rats are less successful at retrieving food pellets with the forelimb ipsilateral to the SCI (Li et al. 1997). Consequently, it seems that impairment in reaching following cervical hemisection is due to the CST injury and not the damage to the ascending pathways of the dorsal columns.

1.2 General pathophysiology following SCI

Information regarding the pathophysiology of human SCI is limited, but various studies indicate that there are strong correlations between human and rodent spinal injuries (Metz et al. 2000; Cheriyan et al. 2014). The similarities between the human and rodent inflammatory response (Fleming et al. 2006) and the morphological response (Norenberg et al. 2004) after SCI permit useful translation to clinical use of treatments developed in animals. Experimental rat SCI models are also similar to the human SCI with respect to motor function recovery related to electrophysiological changes, such as motor evoked potentials and somatosensory evoked potentials, and lesion size assessed using MRI examination (Metz et al. 2000).

The pathophysiological mechanism of SCI is more than a simple mechanical disruption of nerve transmission following injury. In addition to the primary lesion of the spinal cord, a multi-step cascade results in progressive enlargement of the injury, due to factors that include an inflammatory reaction, ischaemia, oedema, haemorrhage and cytotoxicity (Ronsyn et al. 2008). Although there is little or no loss of interneurons and motor neurons several segments beyond the injury, there are significant changes in their biochemical, and consequently physiological, properties (Petruska et al. 2007; Button et al. 2008; Roy et al. 2012). Thus, it is essential to use reliable animal models to assess the pathophysiological mechanisms of SCI and to investigate neuroplasticity in the intact spinal cord caudal to the lesion site. Animal models are also important for assessing novel therapeutic strategies. The pathological sequelae following acute SCI are divided into two broad chronological events: the primary injury and the secondary

injury, due to the additional damaging processes initiated by the primary injury (Taoka et al. 1998; Dumont et al. 2001; Priestley et al. 2012).

1.2.1 Primary injury

The primary injury corresponds to the immediate mechanical damage to neuronal structure, leading to a haemorrhagic zone of necrosis in the grey matter. Cells, especially neurons and their axons, become permeabilized acutely following injury due to compressive and shear forces. Animal studies have demonstrated that neurological impairment increases relative to the force of trauma and the duration of compression. Basically, there are several types of primary injury clinically. The most common mechanism involves impact plus persistent compression (Tator 1996). This insult happens in burst fracture with bony fragment compression of the spinal cord, fracture-dislocation and disc rupture following injury. Another mechanism of primary injury is caused by flexion, extension, rotation, or dislocation producing shearing or stretching of the spinal cord and/or its blood supply. This type of injury may be present without obvious radiological evidence of trauma, but it is common in adults with underlying degenerative spine disease (Tator 1996). Missile injury, sharp knife cutting and severe distraction leads to laceration and transection injury, which is another type of mechanism. This type of injury may occur to varying degrees in SCI, from minor injury to complete transection. The primary mechanical injury also serves as the nidus from which additional secondary mechanisms of injury extend. Consequently, the damage can spread from the lesion epicenter to caudal and rostral segments.

1.2.2 Secondary injury

Following the primary physical damage and death of neural cells, the secondary phase of the injury begins. This response of SCI can be divided into three phases, an acute phase, subacute phase and a chronic phase (Tator 1995; Bareyre et al. 2003).

Typically, the acute phase represents the first 24 to 48 hours following injury. The phase is characterized by vascular dysfunction, including disruption in blood flow leading to local infarction caused by hypoxia and ischaemia (Dumont, Okonkwo et al. 2001). Oedema and haemorrhaging, which correlate with injury severity, result in additional necrotic cell death (Fig 1.1). Microglia, responding to by-products of necrosis (DNA, ATP, K+), become activated and secrete inflammatory cytokines that act to recruit systemic inflammatory cells. Injury leads to increased intracellular sodium and calcium ions due to failure of ion pumps, inactivation of ion channels, reverse function of ion exchangers, and membrane depolarization (Stys et al. 1998). Excessive intracellular Ca^{2+} results in neuronal cell death and axonal degradation through activation of proteases and mitochondrial dysfunction. Calpains are activated after injury and lead to degradation of cytoskeletal proteins, such as neurofilaments and microtubules, that cause axon dysfunction (Banik et al. 1997). Increased production of reactive oxygen species (ROS) occurs following SCI because of mitochondria dysfunction caused by metabolic imbalances and excess intracellular Ca^{2+}. ROS include superoxide and hydrogen peroxide that can cause apoptosis of oligodendrocytes and neurons (Mronga et al. 2004).

The subacute phase is a period that lasts 2 days to 2 weeks following injury in animal models of SCI. In humans, it likely lasts from 2 weeks to 6 months. This phase is

characterized by excessive release of excitatory neurotransmitters, massive immune cell infiltration, delayed cell death, and demyelination/degeneration. SCI leads to a strong inflammatory response, with the recruitment of peripherally derived immune cells such as neutrophils (6-24 hours), macrophages (24 hours to 2 weeks) and T cells (Bethea et al. 2002). In addition to massive ischaemic necrosis, apoptosis has also been identified as a significant event in the injury pathophysiology, especially for vulnerable neurons and oligodendrocytes (Liu et al. 1997).

In the chronic phase, continued activation of the immune system, apoptotic cell death, channel and receptor dysfunction, and demyelination accompany Wallerian degeneration and are ongoing in perilesional areas (Bareyre et al. 2003). There is some attempt by CNS axons to sprout after injury but the newly formed growth becomes dystrophic (Kerschensteiner et al. 2005) after exposure to the inhibitory extracellular matrix molecules (Fitch et al. 2008). Growth-associated inhibitors such as myelin associated glycoproteins, Nogo and oligodendrocyte myelin glycoprotein are also expressed in the vicinity of the lesion and hinder any axonal growth (Sekhon et al. 2001; Filbin 2003). All these processes contribute to conduction deficits.

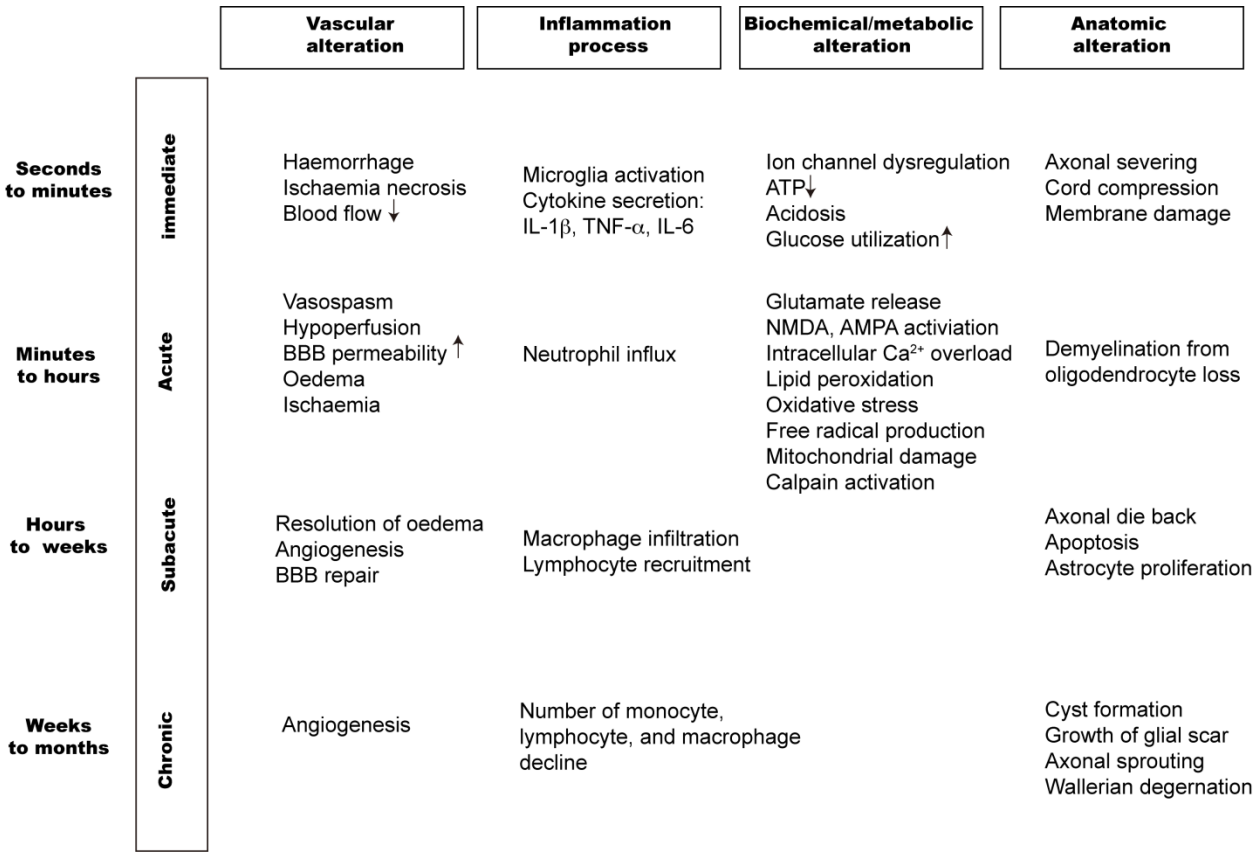

Figure 1-2 Pathophysiological processes following SCI.

The timeline summarizes the phases after SCI. The events that occur after SCI are divided into the immediate (seconds to minutes), acute (minutes to hours), subacute (hours to weeks), and chronic (weeks to months). The phases are characterized by vascular alterations, inflammation processes, biochemical/metabolic alterations, and anatomical change (adapted from (Bareyre et al. 2003; Priestley et al. 2012; Siddiqui et al. 2015).

1.3 Spontaneous functional recovery following SCI

There are many studies showing that some degree of functional recovery occurs after SCI in both humans and rodents, particularly after partial, incomplete injuries (Weidner et al. 2001; Tetzlaff et al. 2009). This finding indicates that reorganization throughout the neuraxis can compensate for the disrupted pathways and rebuild a circuit for control of motion. The processes underlying the diverse neurological changes are summarized by the term “neuroplasticity”. Although this neuroplasticity has been related to spontaneous functional recovery (Onifer et al. 2011), the underlying mechanisms are still not fully characterized.

1.3.1 Molecular and cellular changes following SCI guide neuroplasticity

Following injury, even though successful regeneration does not occur in the CNS, neurons are capable of mounting a transient regenerative response, as evidenced by the expression of regeneration associated proteins and genes (Kruse et al. 2011). The most commonly upregulated genes and transcription factors that are found to be associated with regeneration are: c-jun, activating transcription factor 3 (ATF-3), heat shock protein 27 (HSP-27), growth associated protein – 43 (Gap-43), signal transducer and activator of transcription – 3 (STAT3) (Sun et al. 2010). This indicates that the intrinsic growth program inherent to neurons is activated in response to injury; however, it is not sustained. The upregulation of molecules that contribute to axonal regrowth, such as neuronal calcium sensor-1 (NCS-1) (Yip et al. 2010) and mammalian target of rapamycin (mTOR) (Liu et al. 2010), can induce compensatory sprouting from unlesioned nerve pathways. A number of the molecules that can guide growing axons in

the developing nervous system are also present in the injured spinal cord (Jacobi et al. 2014). These factors can support growing collaterals and initiate synaptogenesis during circuit remodelling.

1.3.2 Reorganization of descending pathways after SCI

Recently, it has been recognized that spontaneous and treatment-induced functional recovery is correlated to sprouting of lesioned and spared descending axons (Fouad et al. 2001; Weidner et al. 2001; Bareyre et al. 2004). It has been demonstrated using secondary lesions (Weidner et al. 2001; Kanagal et al. 2009), electrophysiology, and retrograde neurotracing (Bareyre et al. 2004) that the new neuronal circuits make functional connections. Adult neurons in the CNS cannot regenerate over long distances, but axotomized neurons can form short sprouts from their damaged axons (Fawcett et al. 1998). The injury provides a stimulus for intact interneurons, white matter tracts and sensory neurons to replace inputs to the spinal neurons that have lost synapses. Collateral or regenerative sprouting of intraspinal axons after SCI may provide an opportunity to make new connections or to strengthen existing synapses on denervated spinal neurons (Brown et al. 2012). A probable mechanism that leads to collateral formation is the upregulation of diffusible attracting factors by the denervated tissue. One study showed that the reorganization of CST circuits is guided by brain derived neurotrophic factor (BDNF) secretion by the target interneurons. Furthermore, knockdown of BDNF in spinal neurons diminished the formation of new CST pathways (Ueno et al. 2012).

1.3.3 Modulation of the synaptic transmission of individual neurons

The function of the nervous system critically relies on the establishment of precise synaptic connections. There are three different mechanisms able to alter neural activity: (1) modifying the strength or efficacy of synaptic transmission at pre-existing synapses, (2) eliciting the growth of new synaptic connections or the pruning away of existing ones, or (3) modulating the excitability properties of individual neurons (Crupi et al. 2013). SCI denervates spinal neurons by disrupting ascending and descending pathways to and from the brain as well as output to lower motor neurons. The loss of part or all synaptic inputs results in structural modifications in the number, size, and distribution of synaptic contacts on interneurons and motor neurons (Raineteau et al. 2001).

One way that synaptic changes after SCI have been detected is by comparing the synaptophysin level in intact versus lesioned spinal tissues (Nacimiento et al. 1995). Synaptophysin is a protein within synaptic vesicles in the presynaptic terminal. The expression of synaptophysin is decreased after a spinal cord lesion, but the level recovers to normal after a few weeks. This finding suggests that new synapses form on motor neurons following SCI, and this formation could arise from interneurons or sensory afferents transmitting tactile and proprioceptive signals.

Synaptic efficiency could be modified by neurotransmitter changes within neuronal networks. Gamma-aminobutyric acid (GABA) is a widely distributed inhibitory neurotransmitter in the spinal cord and plays a “counter balance” role against enhanced synaptic transmission in the spinal cord as a result of glutamate-mediated excitation of neurons following SCI. GABAergic pathways play the principal role in reducing neuronal

excitability throughout the nervous system. GABA has a presynaptic inhibitory action on primary afferents and on postsynaptic membranes of interneurons and motor neurons (Alvarez et al. 1996). After complete SCI, GABA neurotransmission is increased in the spinal cord below the injury site, as indicated by an increase in GABA synthesizing enzymes, resulting in altered inhibition during the postlesion period (Tillakaratne et al. 2000). These changes in synaptic efficiency and neurotransmission may be important during the period of postlesion recovery.

1.3.4 Reorganization of cortical areas after SCI

Following traumatic SCI axonal architecture is disturbed and the brain grey matter becomes atrophic (Jurkiewicz et al. 2006; Freund et al. 2011; Henderson et al. 2011; Rao et al. 2013). However, in response to SCI, the sensorimotor cortex can undergo a dramatic reorganization. Remodeling of the S1 map correlates with tactile discrimination performance (Xerri et al. 2005; Rao et al. 2013), while different populations of cortical neurons in M1 cortex selectively contribute to the execution and regulation of movements during locomotion in cats (Drew et al. 2002). This cortical reorganization is sometimes found to be compatible with functional recovery (Schmidlin et al. 2004; Rao et al. 2013). Several studies indicate that cortical territories controlling intact body parts tend to enlarge and invade cortical areas that have lost their peripheral targets.(Franchi et al. 2004; Schmidlin et al. 2004; Martinez et al. 2010). Although the underlying mechanism of cortical reorganization is not well clarified, some studies have suggested these results are partly due to changes in neuronal circuits, such as axonal sprouting (Bareyre et al. 2004; Schmidlin et al. 2004; Martinez et al. 2010).

1.4 Pharmacological neuroprotective intervention for SCI

A substantial number of studies have been devoted to the development of new pharmacological therapies in the treatment of SCI. One promising approach in pre-clinical testing is a treatment given immediately after the injury to achieve better neurological recovery. Neurological efficacy in such an experimental setting suggests that the therapy has a neuroprotective role in SCI. The concept of neuroprotection is based on limiting the evolution of secondary damage due to pathophysiological processes following acute SCI and maximizing the extent of uninjured spinal cord tissue. Currently, there is a lack of clinically accepted pharmacological treatment for SCI. This following section will briefly highlight some of the pharmacological neuroprotective treatments which have undergone clinical trials in traumatic SCI patients.

Methylprednisolone

Methylprednisolone was the only clinically approved drug for SCI until 2013. Experimental studies showed that its neuroprotective effect may be due to inhibition of oxygen-induced lipid peroxidation (Hall 1992), and reduction of ED1-positive cells, leading to spinal tissue protection, and reduced posttraumatic axonal die back via the NFκB pathway (Oudega et al. 1999). The National Acute Spinal Cord Injury Study recommended treatment with methylprednisolone for either 24 or 48 hours, within 8 hours after SCI (Bracken et al. 1997). However, based on new Level 1 recommendations in 2013 by the American Association of Neurological Surgeons (AANS) and the Congress of Neurological Surgeons (CNS) (Hurlbert et al. 2013),

methylprednisolone should not be given in the treatment of acute SCI, because of associated harmful side effects such as a higher infection rate, respiratory complications, gastrointestinal haemorrhage and even death (Molano Mdel et al. 2002). However, there still remains controversy regarding the new AANS/CNS guidelines. They suggest methylprednisolone could be applied to patients with cervical SCI undergoing decompression, based on a Cochrane meta-analysis showing some benefit of treating these patients (Bracken 2012; Fehlings et al. 2014). Therefore, it becomes important to further investigate the application of steroids in SCI patients.

Minocycline

Minocycline, a broad spectrum tetracycline antibiotic, has been shown to have neuroprotective effects through the inhibition of M1 macrophage/microglia (Festoff et al. 2006; Kobayashi et al. 2013). The drug also decreases formation of ROS, reduces matrix metalloproteinase activity and inhibits apoptotic cell death (Yong et al. 2004). Minocycline is an attractive drug for clinicians because it is known to be well tolerated in human, where it has been used for acne treatment for decades.

A randomized Phase II trial has demonstrated that treatment with minocycline leads to a trend to improve functional outcome in patients with cervical motor-incomplete injury, although the improvement was not statistically significantly. The study also showed no serious adverse events in patients receiving treatment (Casha et al. 2012). At present, two Phase III clinical trials are in progress for patients with acute SCI. (NCT01828203, NCT01813240 identified in ClinicalTrials.gov).

Riluzole

Riluzole is a sodium channel blocker, which is FDA-approved for amyotrophic lateral sclerosis (ALS) patients. Several SCI animal studies have reported that sodium channel blockers can attenuate secondary damage by preventing excessive influx of sodium and calcium, which triggers pathological extracellular release of the excitatory neurotransmitter glutamate, leading to cell death (Kobrine et al. 1984; Teng et al. 1997). Riluzole may also exert its neuroprotective effects through neurotrophic factor upregulation (Mizuta et al. 2001; Katoh-Semba et al. 2002). In pre-clinical studies, riluzole-treated animals also had greater preservation of white matter, better mitochondrial function, somatosensory-evoked potentials, and motor neurons following thoracic SCI (Schwartz et al. 2001). Riluzole was also found to have neuroprotective efficacy when the intervention was delayed 1 and 3 h post-injury and continued for 7 days after injury, in a cervical SCI animal experiment (Wu et al. 2013). In the first clinical trials of riluzole for acute SCI, the results showed some functional improvements in cervical SCI patients treated with riluzole, and no complications and side effects were noted (Grossman et al. 2014). Currently, a prospective, randomized, multicenter Phase II/III trial is ongoing in patients with acute C4-C8 SCI (NCT01597518 identified in ClinicalTrials.gov).

Magnesium

Magnesium has been investigated in animals as a neuroprotective agent for traumatic brain injury (TBI) and SCI (Suzer et al. 1999; Kaptanoglu et al. 2003; Gok et al. 2007; Hoane 2007; Wiseman et al. 2009). The biological rationale for magnesium in SCI

includes the attenuation of excitotoxicity through a voltage-dependent blockade of NMDA receptors (Zhang et al. 1996), and the replacement of depleted magnesium levels following injury (Vink et al. 2000). In acute SCI animal models, magnesium sulfate administration has been shown to decrease membrane damage, protect axonal function and improve electrophysiology and behavioural outcome (Suzer et al. 1999; Kaptanoglu et al. 2003). However, the neuroprotective effect was achieved with extremely high doses of magnesium that far exceed the tolerable human dose. More recently, magnesium chloride formulated within polyethylene glycerol (PEG) has been developed, and the dose of magnesium is similar to the clinically-acceptable dose. This formula has been investigated as a potential neuroprotective agent for SCI in thoracic and cervical SCI animal models (Kwon et al. 2009; Lee et al. 2010). A clinical trial of a proprietary form of PEG with magnesium chloride, AC105 (NCT01750684 identified in ClinicalTrials.gov), is currently underway to determine the efficacy of the drug in patients who have a SCI.

Fibroblast growth factor

Fibroblast growth factor-2 (FGF-2; or basic fibroblast growth factor) is a protein that has been shown to promote neurological functional recovery by rescuing neurons adjacent to the lesion site, promoting angiogenesis and a reduction in cavitation (Teng et al. 1999; Kang et al. 2010; Kang et al. 2013). The underlying mechanism is not well defined. A recent study suggested that FGF-2 activates the downstream signal PI3K/Akt, which is correlated to neuronal survival and regeneration (Zhang et al. 2013). A Phase II randomized, clinical trial for patients with acute cervical SCI began in 2012

(NCT01502631 identified in ClinicalTrials.gov).

Rho protein antagonist

Rho is a small intracellular GTPase. After SCI, myelin-associated inhibitors and CSPG can activate the Rho signaling pathway. Rho activation in neurons has been shown to result in neuronal growth cone collapse, neurite retraction, and cell body rounding (Jalink et al. 1994). In experimental studies, the activity of the Rho/Rho kinase pathway is enhanced, contributing to increased levels of apoptosis in neurons, astrocytes and oligodendrocytes (Dubreuil et al. 2003; Sung et al. 2003).

Recent evidence suggests that the inactivation of Rho allows neurons to enhance axon growth on growth inhibitory substrates (Jain et al. 2004; McKerracher et al. 2006). However, there are some studies that report Rho inhibition exerting neuroprotective effects after SCI (Dubreuil et al. 2003; Lord-Fontaine et al. 2008). C3 transferase (C3), an enzyme from *Clostridium botulinum*, has the ability to block Rho function. In a mouse model of SCI, C3 treatment enhanced axonal regeneration and improved motor function (Dergham et al. 2002). In addition, C3 treatment can significantly reduce the number of apoptotic cells and increase tissue sparing following SCI (Dubreuil et al. 2003; Lord-Fontaine et al. 2008), suggesting that C3 has neuroprotective effects. A phase I/IIa clinical trial has been conducted to investigate the safety and pharmacokinetics of the Rho pathway antagonist, Cethrin® (BA-210) (Fehlings et al. 2011). BA-210 is a recombinant fusion protein composed of C3 and a transport sequence that aids the proteins' penetrative ability to cross cellular membranes in combination with a fibrin

sealant. Although the number of patients was small and there was no control group, these results suggest that BA-210 contributes to functional recovery following SCI without adverse effects. A phase IIb/III clinical trial has been planned for subjects with acute cervical SCI (NCT02053883 identified in ClinicalTrials.gov).

Additional pharmacological therapies that have been explored in human SCI trials but have not demonstrated significant clinical efficacy include: naloxone (Bracken et al. 1990), thyrotropin releasing hormone (Pitts et al. 1995), trilazad mesylate (Bracken et al. 1997), nimodipine (Pointillart et al. 2000) GM-1 ganglioside (Geisler et al. 2001), gacyclidine (Lepeintre et al. 2004), and granulocyte colony stimulating factor (Yoon et al. 2007).

Because of the limited effective treatments after SCI, it is not surprising that many pharmacological and non-pharmacological approaches (e.g. systemic hypothermia, rehabilitation) are being investigated to provide better treatment for SCI patients. Although therapeutic effects have been observed in preclinical work, we have not seen any treatments translated successfully into clinical use. One of the reasons for this is the complexity and multi-factorial nature of the secondary pathophysiological change following SCI. Most treatments only target one aspect of this response; however, a successful approach is likely to depend on a combination of different treatments.

1.5 Strategies promoting neuroplasticity following SCI

The existence of spontaneous structural and functional plasticity in the CNS following SCI raises the possibility that this could be a therapeutic target for enhancing functional recovery. However, this endogenous effect is often suboptimal and highly variable, and new strategies need to be developed to enhance the process of plasticity in order to maximise the potential for recovery. In order to obtain efficient functional recovery, these strategies may involve treatment to strengthen existing connections or improve nerve conduction in axons that survived but are functionally impaired, or promotion of collateral sprouting of either damaged or spared pathways which can lead to the formation of new neuronal pathways which can pass around the lesion site (Fig 1.2). It is thought that the development of novel therapeutic approaches for promoting meaningful neuroplasticity changes following SCI could involve several strategies.

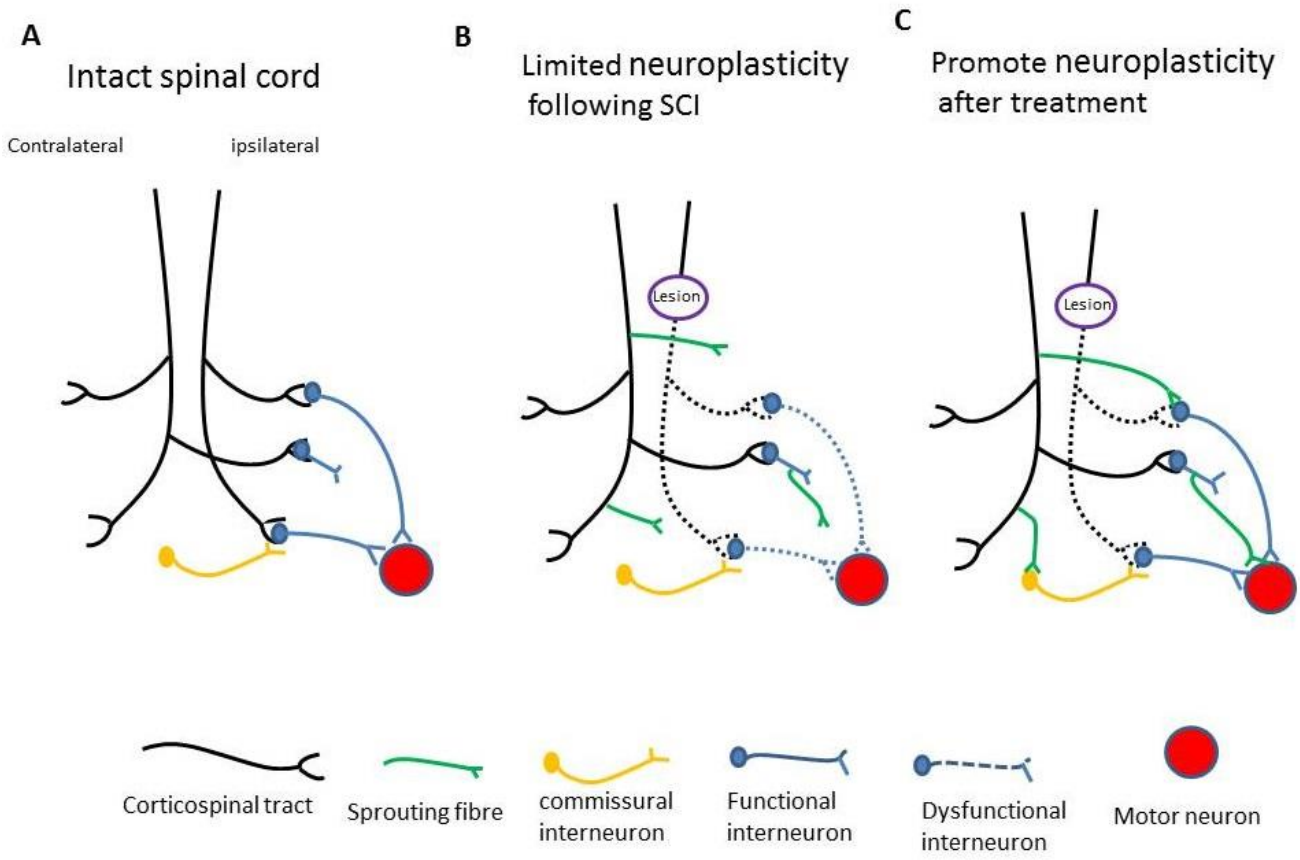

Figure 1-3 Neuroplasticity changes in spinal cord after SCI

(B) Aberrant and non-functional sprouting in injured or intact CST. (C) After neuroplasticity promoting treatment, sprouting contralateral CST axons cross the midline and rebuild functional contacts with interneurons, which bypass the lesion site.

1.5.1 Increase intrinsic regenerative ability

There have been several novel methods to increase the intrinsic regenerative ability of neurons. To promote axonal sprouting and remodelling, one generally used strategy is the application of exogenous neurotrophic factors, such as BDNF and neurotrophin-3 (NT3) (Lu et al. 2008; Fouad et al. 2013). Following pyramidotomy, transduction of spinal motoneurons with NT3 resulted in a significant sprouting of fibres across the

midline towards the motor neurons (Zhou et al. 2003). Sustained expression of BDNF and NT3 in the cortex and spinal cord, respectively, further enhanced the axonal sprouting from the intact side in animal models of SCI (Zhou et al. 2003; Fouad et al. 2013). Injection, or grafts of genetically modified cells which can secrete neurotrophic molecules, or viral delivery systems, can be used to deliver neurotrophic factors to the lesion (Lacroix et al. 2000). Work from other laboratories revealed that administration of cyclic adenosine monophosphate (cAMP) influences intracellular signalling pathways and can promote CNS regeneration (Hannila et al. 2008). An alternative approach is the manipulation of key regulatory genes involved in the intrinsic growth program. Genetically overexpressing factors such as STAT3 (Lang et al. 2013), NCS-1 (Yip et al. 2010) and mTOR (Liu et al. 2010) has been reported to stimulate spared fibres sprouting into the denervated side, which promoted functional recovery. The approach to upregulate the intrinsic growth potential of a neuron would be a promising strategy to promote neuroplasticity following SCI.

1.5.2 Cell based transplantation

Key experiments using peripheral nerve grafts, rich in Schwann cells, demonstrate that CNS neurons do retain the capacity to mount a regenerative response following SCI (David et al. 1981). Several transplanted cell types contribute to anatomical neuroplasticity by greatly increasing axonal regeneration and fibre density in the injured spinal cord. For example, Schwann cell and olfactory ensheathing glia (OEG) grafts promote extensive axonal growth and elongation (Xu et al. 1995; Ramon-Cueto et al. 1998) with axonal sprouting (Guest et al. 1997). The transplanted cells can modify the

peri-lesional environment. Gliosis and astrocyte reactivity have been shown to be reduced with transplantation of OECs in acute SCI (Lakatos et al., 2003; López-Vales et al., 2006) and of bone marrow stem cells in acute and chronic SCI (Lu et al., 2007). One essential role of stem and associated cell transplantation for SCI lies in the considerable capacity of trophic mediator secretion, promoting neuroplasticity (Teng et al. 2002).

1.5.3 Inactivation of the growth-inhibitory extracellular environment

The intrinsic barrier to growth, attributed to the decline in neuroplasticity of CNS neurons as they mature, affects the capability of injured CNS neuron to both grow and remodel. Neutralization of the inhibitory environment that is formed after injury is one approach used to promote neuroplasticity (Maier et al. 2006). One major barrier to axonal regeneration and plasticity in the injured CNS in the presence of growth-inhibitory factors associated with CNS myelin (Gonzenbach et al. 2008; Xie et al. 2008). A wide spectrum of inhibitors has been observed to limit neurite outgrowth in vitro, such as Nogo (Chen et al. 2000), myelin-associated glycoprotein (McKerracher et al. 1994) and oligodendrocyte myelin glycoprotein (Wang et al. 2002). When these inhibitors are antagonized in animal models of SCI, there is significant regeneration of severed axons in vivo. Recently, numbers of studies demonstrated uninjured axons could sprout robust collateral axonal processes in the absence of myelin inhibitory factors. In rodent models of SCI, deletion of Nogo-A leads to a significant increase in collateral sprouting that crosses the midline and correlates with significant functional recovery (Kim et al. 2003; Simonen et al. 2003). Moreover, in primate models of SCI, anti-Nogo-A antibodies can significantly improve functional recovery, that correlates with an increase in sprouting of

CST collaterals, but not with CST regeneration (Fouad et al. 2004; Freund et al. 2006). These results suggest that non-regenerative anatomical plasticity is a substrate for functional recovery under conditions of myelin protein inhibition.

After CNS injury, in order to stabilize the site of injury, a glial scar forms at the injury site and this leads to a physical and molecular barrier to axonal growth (Fitch et al. 2008). Chondroitin sulphate proteoglycans (CSPGs) are a major class of growth-inhibitory molecules associated with the glial scar extracellular matrix, and they are densely upregulated following injury to the CNS (Galtrey et al. 2008). The distribution of CSPGs within the glial scar closely correlates with the failure of axonal growth in the injured spinal cord in experimental studies. Treatment of the spinal cord with the bacterial enzyme chondroitinase ABC (ChABC) is the best established and most widely used mechanism through which CSPGs have been degraded in order to diminish their inhibitory properties (Bradbury et al. 2011). In a dorsal column crush animal model, ChABC treatment enhanced the sprouting of intact fibres from the ventral CST and increased innervation of denervated grey matter (Bradbury et al. 2002). Serotonergic fiber sprouting was also observed following ChABC treatment in rat (Garcia-Alias et al. 2009) and cat (Tester et al. 2008) experimental studies. Taken together, mounting evidence suggests that removal of growth inhibitory factors plays a role in promoting axon plasticity after SCI.

In addition to pharmacological intervention, activity-dependent approaches, such as physical rehabilitation, may contribute to functional recovery by enhancing the

rebuilding, strength, and selection of synapses. Recent studies have also focused on combinatorial therapies based on pharmacologically promoting structural plasticity combined with a rehabilitation program. The results revealed that this is a more effective strategy to enhance functional recovery compared to either pharmacological treatment or rehabilitative training alone (Ying et al. 2008; Garcia-Alias et al. 2009).

1.6 Benefits of rehabilitation

A well–establish therapeutic approach to promote neuroplasticity and recovery following SCI is rehabilitative training (Beekhuizen 2005; Edgerton et al. 2006). The capacity for CNS reorganization is a well-documented property that may contribute to many examples of functional recovery. It is now widely accepted that rehabilitation training is the current successful treatment to promote neuroplasticity following SCI with/without administration of pharmacological agents. I summarize below some of the benefits gained from rehabilitation following SCI.

1.6.1 Up-regulation of growth/plasticity associated factors

Neurotrophic factors are a class of polypeptide growth factors that promote survival, growth and maintenance of neurons, and modulate synaptic strength to improve signal transduction. After SCI, the expression of neurotrophic factors is downregulated but it increases with training and voluntary locomotor programs (Dupont-Versteegden et al. 2004; Hutchinson et al. 2004; Ying et al. 2008). Exercise-induced increase in neurotrophic factors in the damaged spinal cord can promote regeneration of damaged axons, the plasticity of spinal cord circuity and improve functional locomotor recovery. For example, BDNF (Gomez-Pinilla et al. 2002; Boyce et al. 2007), NT3 (Grill et al. 1997), and insulin-like growth factor 1 (Jung et al. 2014) have been shown to enhance survival of damaged neurons, modulate synaptic strength and contribute to the recovery of function following injury. Notably, the effects of activity-dependent rehabilitation are not limited to the region of the spinal cord most likely stimulated by training, but also increases some neurotrophic factors throughout the spinal cord (Cote et al. 2011).

1.6.2 Contribution to plasticity in lesioned and spared tracts

Various studies have demonstrated that spontaneous sprouting of lesioned and spared descending axons is linked to functional recovery following SCI (Weidner et al. 2001; Bareyre et al. 2004). Recently, some studies have demonstrated that rehabilitative training in a reaching task increases spontaneous collateral sprouting of CST axons after CNS lesion (Girgis et al. 2007; Starkey et al. 2011). In addition to CST axonal sprouting, MiniMitter running wheel exercise can also induce increased serotonin fibre density in the lumbar spinal cord of mice after moderate contusion injury (Engesser-Cesar et al. 2007). It has been suggested in SCI models that treadmill training activates cellular and molecular growth factors within the spinal cord (Shin et al. 2014). These plasticity-promoting factors might contribute to axonal sprouting and functional recovery following training. Another important issue that should be considered is that guidance of new sprouting fibres to appropriate post-synaptic cells represents a complex, delicate process, which is still poorly understood. Once new neuronal circuits have formed, the rehabilitation training may favour appropriate projections by modulating and stabilizing the neuronal pathways, thus boosting functional improvement.

1.6.3 Change in neuronal properties can facilitate recovery

It is well-known that SCI induces changes in motor neuron activity patterns due to the interruption of descending supraspinal and propriospinal pathways. Removal of descending presynaptic inhibition of group Ia axons may contribute to the decrease in reflex threshold and loss of habituation by motor neurons (Hochman et al. 1994). Recent data revealed that motor neurons can change their properties as a response to

SCI and rehabilitation. Passive cycling exercise can moderate the deleterious responses of motor neurons to SCI and promote structural and physiological plasticity in motor neurons below the lesion site (Beaumont et al. 2004). Exercise also modifies the activity of inhibitory transmitters after SCI. Levels of the inhibitory neurotransmitter, glycine, and GAD-67, a synthetic enzyme for GABA, are both increased at chronic time points after SCI (Tillakaratne et al. 2000; Khristy et al. 2009). This adaption in inhibitory tone may indicate a compensatory response to the loss of descending inhibition. However, both glycine and GAD-67 levels and the expression of $GABA_B$ receptors are restored to normal levels by treadmill training following spinal cord complete transection (de Leon et al. 1999).

One electrophysiological study demonstrated that synaptic input and action potential after-hyperpolarization of motor neurons was amplified after treadmill stepping following SCI (Petruska et al. 2007). These changes in motor neuron properties following exercise in SCI could be caused by changes in ion channel activity (Gardiner et al. 2006). Changes in neuronal excitability associated with training represent a promising target for restoring motor function following SCI.

1.6.4 Training facilitates cortical reorganization

The somatosensory cortex serves several key functions in the brain. Cortical map changes following SCI have been reported in patients and in animal models (Cohen et al. 1991; Bruehlmeier et al. 1998; Fouad et al. 2001; Mikulis et al. 2002). Following cervical SCI, expansion of motor maps can be promoted by forelimb reaching training

over several days (Girgis et al. 2007; Krajacic et al. 2009). The enhanced cortical maps modulated by rehabilitation correlate with increasing collateral sprouting of CST fibres and neurological recovery (Fouad et al. 2001). In addition to the motor system, recovery of tactile abilities has also been observed in rats receiving sensorimotor training. However, this recovery was not present in rats without training (Martinez et al. 2009).

1.7 Docosahexaenoic acid

Fatty acids are carboxylic acids with a long unbranched carbon hydrogen tail of varying lengths, which is either saturated or unsaturated. Fatty acids that have carbon-carbon double bonds are known as unsaturated. Fatty acids without double bonds are known as saturated. PUFAs contain multiple double bonds and can be categorized by the position of the first double bond from the methyl end of the acyl chain. According to the international nomenclature, the position of the first double bond is given by the n-x (also ω-x or omega-x) notation, counting the number of carbon atoms from the methyl end. DHA contains 22 carbons and 6 double bonds, and the first double bond occurs at the third carbon atom from the methyl group. It therefore belongs to the class of omega-3 fatty acids.

Mammals are unable to synthesize double bonds between carbons 1 and 9 because they lack the necessary desaturase enzymes (Salem et al. 1989). The long chain PUFAs may be directly ingested from the diet or produced from their shorter chain precursors. A major source of omega-3 PUFAs is cold-water oily fish. DHA is synthesized through desaturation and elongation reactions from a precursor, an 18-carbon fatty acid, alpha-linolenic acid (ALA), which is available in the diet. Although neurons are major targets for DHA accumulation, they are unable to synthesize DHA. The conversion of ALA to DHA occurs primarily in the liver (Igarashi et al. 2007). After release from the intestine after a lipid-rich meal or from the liver, fatty acids circulate in the plasma loosely bound to albumin and cross plasma membranes via fatty acid transporters.

The omega-3 PUFA can directly diffuse into cells and also interact with fatty-acid-transport proteins (FABPs). The intracellular transport of long-chain fatty acids involves binding to FABPs, which recent evidence suggests could be used as markers of organ injury (Michael-Titus et al. 2014). At least 9 members of FABPs have been identified and they have various roles (Furuhashi et al. 2008). Among these, brain FABP (B-FABP) is distinguished from other FABPs by its strong affinity for omega PUFAs, in particular DHA, which indicates that B-FABP may be intricately linked to the role of DHA in the nervous system (Xu et al. 1996; Balendiran et al. 2000). In recent studies, there has been increased interest in the neuroprotective benefits of PUFAs, especially omega-3 PUFAs, which have shown significant therapeutic potential in neurology and in particular for neuroprotection (Lauritzen et al. 2000; Heller et al. 2006; Kidd 2007; Michael-Titus 2007; Dyall et al. 2008).

1.7.1 Molecular targets of DHA

An extensive literature has demonstrated that omega-3 PUFAs have multiple actions. To explain the effects on DHA, a number of explanations have been formulated, ranging from unspecific effects on cell membrane fluidity to specific binding to protein targets. Dietary supplementation with DHA may affect intracellular and intercellular signalling as well as physical membrane properties (Farooqui et al. 2004; Fukaya et al. 2007). DHA can also modulate neurological function directly or indirectly at many different levels through various membrane proteins, such as enzymes, ion channels, nuclear receptors, and G-protein coupled receptors (Fig 1.3). A comprehensive list of the targets of DHA is

yet to be produced. However, there are several strong candidates that have been identified as possible targets involved in DHA effects.

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1.7.1.1 Involvement of ion channels

PUFAs have beneficial effects in both epilepsy and cardiac arrhythmia due to their effect on voltage-gated ion channels (Leaf et al. 2003; Taha et al. 2010). In neuronal tissue, a role for PUFA modulation of ion channels has been suggested in synaptic plasticity, development, and in the outcome of ischaemia, seizures, and perhaps other pathological conditions (Bazan 2006). DHA treatment could reduce the glutamate excitotoxity associated with SCI and TBI through modulation of voltage-gated ion channels. Both in vivo and in vitro studies also demonstrate that omega-3 PUFAs suppress neuronal excitability by inhibiting voltage-sensitive Na^+ and Ca^+ channels (Vreugdenhil et al. 1996; Voskuyl et al. 1998; Leaf et al. 1999), and by activation of the two-pore domain background K^+ channel, TREK-1 (TWIK-related potassium channel-1) (Boland et al. 2008).

1.7.1.2 Involvement of intracellular fatty acid-binding proteins

Fatty acid-binding proteins (FABPs) are a multigene family of cytosolic proteins that are the counterpart to extracellular albumin, which can facilitate the transfer of fatty acids across extra- and intracellular membranes (Chmurzynska 2006). There are a total of nine FABPs, but only Heart-FABP (H-FABP), Epidermal-FABP (F-FABP), and Brain-FABP (B-FABP) have been identified in the nervous system (Myers-Payne et al. 1996;

Owada et al. 1996). H-FABP expression increases in gray matter during development (Owada et al. 1996). The concentration is highest in the synaptosomal cytosol, which suggests H-FABP has an important function at the synapse (Pu et al. 1999). E-FABP is highly expressed during neurogenesis, neural migration and differentiation of neurons (Allen et al. 2000; Liu et al. 2000). B-FABP is expressed mainly in glial cells at early stages of brain development and its level decreases in neonatal and adult brain (Owada et al. 1996). B-FABP may be an important target to explore, since it has high affinity for DHA (Balendiran et al. 2000).

1.7.1.3 Involvement of transcription factors

DHA can also activate transcription factors, such as retinoid X receptors (RXRs), and peroxisome proliferator-activated receptors (PPARs), to alter gene expression. RXR is an obligatory component of various nuclear receptor heterodimers involving the retinoic acid receptor (RAR), the vitamin D receptor, the thyroid hormone receptor and PPARs (Calderon et al. 2007). It has been shown that DHA can bind to RXRs and influence RXR-mediated transcription in brain (de Urquiza et al. 2000; Lengqvist et al. 2004). RXRs are activated and expressed by microglia after contusion SCI (Schrage et al. 2006). One of the major transcription factors responsible for the regulation of inflammatory cytokines is NFκB. A previous study has shown that RXR inhibits NFκB-dependent gene expression and reduces interleukin-12 production in macrophages (Na et al. 1999). Thus, DHA may reduce the inflammatory response by modulating reactive microglia.

Omega-3 PUFAs can activate PPARs, which include three isotypes: PPARα, PPRAγ, and PPARδ/β. Various experimental studies have shown that PPAR agonists exert neuroprotective effects through PPARγ activation (Sundararajan et al. 2005; Zhao et al. 2006; McTigue et al. 2007; Yi et al. 2008). An *in vitro* study suggests that EPA and DHA may reduce lipopolysaccharide-induced nuclear factor-kappaB (NF-κB) activation and monocyte chemoattractant protein-1 (MCP-1) expression via activation of PPARγ (Li et al. 2005). In addition, omega-3 PUFAs supplementation appeared to increase PPARs as well as RXR expression in aged rats' brain compared to an aged untreated group (Dyall et al. 2010). The effect may be linked to the neurogenesis promoting effect of DHA seen in *in vitro* and *in vivo* studies (Kawakita et al. 2006).

Activation of PPARs can also increase BDNF and NGF levels (Meng et al. 2011) and induce neuronal differentiation by modulating the BDNF/TrkB pathway (D'Angelo et al. 2011). An increased BDNF level was also detected in TBI (Wu et al. 2004; Wu et al. 2011) and cerebral ischaemia (Blondeau et al. 2009) animal models after omega-3 PUFAs supplementation and was correlated with neurological functional recovery. These findings suggest that DHA can upregulate BDNF expression and promote neurogenesis through PPAR activation.

1.7.1.4 Involvement of G-protein coupled surface receptors

G-protein coupled receptors (GPRs) are known to play important physiological roles in response to fatty acids. GPR120 (Hirasawa et al. 2005) and GPR40 (Itoh et al. 2003) are both able to bind long-chain fatty acids and both activate intracellular signalling pathways. GPR120 is highly expressed in adipose tissue and proinflammatory

macrophages (Oh et al. 2010). By signalling through GPR120, DHA and EPA mediate potent anti-inflammatory responses and inhibit the TNF-α signalling pathway, and this effect is abrogated by GPR120 knockdown (Oh et al. 2010). A recent study also reported that the anti-inflammatory effect of DHA may be related to cPLA2 activation via GPR120 (Liu et al. 2014). In addition, PUFAs also promote the activation of PI3K-Akt pathways via GPR120, leading to an anti-apoptotic effect (Katsuma et al. 2005).

GPR40 is also a receptor for PUFA, and in addition to expression in the pancreas, its gene was reported to be expressed ubiquitously in the human brain (Briscoe et al. 2003). GPR40 has been shown to be localized to β-cells of the pancreas and to modulate insulin secretion in response to free fatty acids, but its role in the CNS is not well clarified. In GPR40 gene transfected rat neuronal stem cell cultures, one study demonstrated that DHA induced neurite growth and neuronal differentiation (Ma et al. 2010). This suggests that PUFAs might act as extracellular signalling molecules at such membrane receptors to regulate neuronal function and promote neuronal differentiation (Yamashima 2008; Ma et al. 2010).

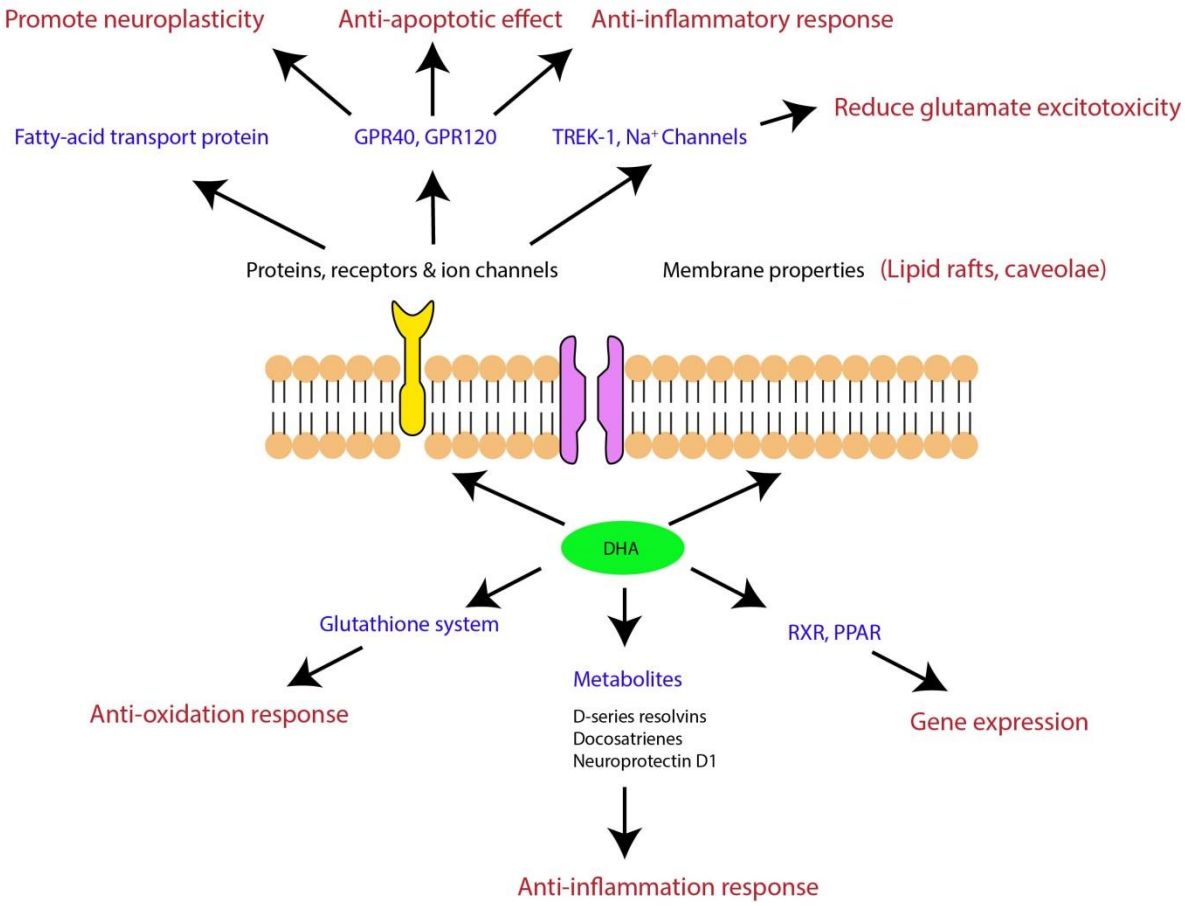

Figure 1-4 Cellular targets and metabolites of DHA

DHA and its metabolites acts through various mechanisms: its incorporation into membranes can change the properties of membrane microdomains (lipid rafts and caveolae); it binds to membrane proteins, receptors and ion channels to activate neuroprotective and neuroplasticity promoting mechanisms; DHA metabolites (resolvins, docosatrienes, and neuroprotectins D1) also contribute to the anti-inflammatory response. Adapted from (Dyall et al. 2008; Michael-Titus et al. 2014).

1.7.2 DHA and neuronal function

The CNS has the second highest concentration of lipids after adipose tissue, with a large proportion of brain lipids being comprised of PUFAs (Salem et al. 1989; Horrocks et al. 2004). PUFAs are major components of glial and neuronal membrane phospholipids, and play a pivotal part in brain membrane remodelling and synthesis, and signal transduction (Rapoport et al. 2007). Both omega-3 and omega-6 PUFAs are important components of cell membranes and are precursors to many other substances in the body such as those involved with regulating blood pressure and inflammatory responses. The major part of omega-3 PUFAs in the CNS is DHA, representing 10-20% of total fatty composition, whereas eicosapentaenoic acid (EPA) and arachidonic acid (AA) represent considerably less (Michael-Titus 2009). Normal CNS function and structure has been proposed to depend on an optimal balance between omega-3 and omega-6 PUFAs and if this balance is disturbed, it may lead to neurologic deficits (Noaghiul et al. 2003) and cognitive changes (Conquer et al. 2000).

DHA levels in the brain are responsive to dietary intake of preformed DHA (Brenna et al. 2007). During omega-3 PUFA dietary deficiency, the transfer of DHA out of the CNS across the blood brain barrier is reduced, while unesterified DHA is also rapidly recycled from brain phospholipids in order to maintain DHA levels (Contreras et al. 2000).

1.7.3 DHA and neuroprotection

The beneficial effect of omega-3 PUFAs following injury to the spinal cord was first documented by Lang-Lazdunski et al. in a rat model of transient spinal cord ischaemia (Lang-Lazdunski et al. 2003). Over the past 10 years, there has been increased interest

in the health benefits of PUFAs, with evidence emerging that omega-3 PUFAs have significant therapeutic potential in a variety of CNS disorders, including Zellweger syndrome, schizophrenia, depression, and Alzheimer's disease (Calon et al. 2007). In particular, a number of clinical and pre-clinical studies have reported neuroprotective effects of diets enriched in omega-3 PUFAs (Morris et al. 2003; Huang et al. 2007; Figueroa et al. 2013).

1.7.3.1 Anti-inflammatory effect

Inflammation is a natural defence response to trauma or microbial invasion. This phenomenon is designed to remove the inflammatory stimulus and rescue tissue from damage. However, an excessive inflammatory response can lead to local tissue damage and further remodelling, which may cause significant injury. Several studies have reported that the functional outcome after SCI is improved by therapies that reduce inflammation (Popovich et al. 1997; Kwon et al. 2004). PUFAs play an important role in the onset and resolution of inflammation. Omega-6 PUFAs, such as AA, contribute to the initiation of inflammation. The primary injury leads to excitotoxicity, which activates cytosolic phospholipase A2 (cPLA2) releasing AA and producing pro-eicosanoids, leukotrienes, prostaglandins, and thromboxanes that mediate inflammation by enhancing vascular permeability, increasing local blood flow and infiltration (Profyris et al. 2004; Jones et al. 2005). Contrary to the omega-6 PUFAs, omega-3 fatty acids, such as DHA and EPA, have well-described anti-inflammatory effects (Mori et al. 2004). Several possible mechanisms have been proposed to explain the anti-inflammatory effects of omega-3 fatty acids. Omega-3 fatty acids play a role in substrate competition,

preventing AA conversion into proinflammatory eicosanoids such as prostaglandins (PGs) and leukotrienes (LTs). Omega-3 fatty acids can serve as an alternative substrate to produce less potent 5-series LTs and 3-series PGs and thromboxanes (Seki et al. 2009). Recently, many studies have also demonstrated that the metabolites of omega-3 fatty acids play a vital part in anti-inflammatory resolution. The resolving inflammatory exudates use omega-3 fatty acids to produce structurally distinct families of signalling molecules- resolvins, protectins and maresins, collectively termed specialized pro-resolving mediators (SPMs) (Serhan 2014). Specific SPMs shorten the resolution interval by limiting neutrophil recruitment and stimulation of macrophage efferocytosis and tissue regeneration (Serhan et al. 2012).

Omega-3 fatty acids also have potent anti-inflammatory effects on the human immune system. In vitro studies have shown that they can inhibit lymphocyte-endothelial cell adhesion to ameliorate the inflammatory response (Khalfoun et al. 1996), and suppress T-cell-mediated immune function (Wu et al. 1998). They can reduce inflammation by modulating the response of reactive microglia (Antonietta Ajmone-Cat et al. 2012).

1.7.3.2 Anti-oxidant effect

Increase in superoxide and hydroxyl radicals has been well documented in spinal cord injuries (Liu et al. 1998; Bao et al. 2004; Liu et al. 2004). One major event leading to direct damage to neuronal and axonal membrane structure and function after SCI is lipid peroxidation (Hall et al. 2004). Furthermore, lipid oxidation can cause microvascular damage and secondary ischaemia, and potentially lead to protein

oxidation (Berlett et al. 1997) and extensive tissue damage and neuron cell death (Xu et al. 2005). In a series of studies from our group, DHA treatment after thoracic SCI has been shown to significantly decrease oxidative stress, including lipid peroxidation, protein oxidation, and RNA/DNA oxidation (King et al. 2006; Huang et al. 2007). It is likely that the reduction of such damage by DHA plays a crucial role in its neuroprotective effects. *In vitro* studies have shown that DHA supplements can reduce oligodendrocyte injury by increasing neuronal glutathione activity and preventing hydrogen peroxide-induced cell death (Brand et al. 2008). Neurofilament proteins are vulnerable to oxidative stress, which results in alteration in their structure and the eventual collapse of the cytoskeleton (Gelinas et al. 2000). The anti-oxidant capacity of DHA may thus also ameliorate the neurofilament loss.

1.7.3.3 Reducing glutamate excitotoxicity

The release of glutamate after traumatic SCI leads to death of neurons and oligodendrocytes (Liu et al. 1999; Park et al. 2004). Work from other laboratories showed that DHA treatment can reduce glutamate-induced excitoxicity both *in vivo* (Hogyes et al. 2003) and *in vitro* (Wang et al. 2003). The underlying mechanism is possibly that DHA inhibits voltage-gated sodium and calcium ion currents, thus reducing the depolarization-induced glutamate efflux (Seebungkert et al. 2002) and N-methyl-D-aspartate (NMDA) receptor activation (Nishikawa et al. 1994). In another possible mechanism, DHA could activate two-pore domain potassium channels, i.e. the Twik-related potassium channel 1 (TREK-1) and the TWIK-related arachidonic acid stimulated potassium channel (TRAAK) (Kanellopoulos et al. 2000). TREK-1 is

expressed at both presynaptic and postsynaptic sites. In glutamate-containing neurons, opening of presynaptic TREK-1 channels will reduce glutamate release by closing voltage-dependent calcium channels. At the postsynaptic level, membrane hyperpolarization will decrease NMDA receptor activation and excitotoxicity in pathological conditions (Franks et al. 2004).

1.7.4 DHA and neuroplasticity

PUFAs are incorporated into membrane phospholipids, thus playing important structural roles, but they can also activate a variety of cell signaling pathways. Our investigation of DHA effects on neuroplasticity was prompted by observations indicating that DHA treatment influences brain plasticity and function. Some studies indicate that DHA provides support to learning and memory in animal models of Alzheimer's disease (Hashimoto et al. 2002; Lim et al. 2005) and brain injury (Wu et al. 2004).

1.7.4.1 DHA enhances synaptic plasticity

DHA has widespread effects on synaptic function, but the mechanism underlying the promotion of synaptic plasticity by DHA remains to be elucidated. However, these functions likely involve a complex interplay of synergistic effects on neuronal membrane structure and function. As mentioned previously, DHA is a key building block for synaptic membranes and significantly alters many basic membrane properties and consequently regulates neurotransmission and signal transduction (He et al. 2009). Oral chronic DHA intake has been shown to promote the synthesis of synaptic membranes, elevate the level of phosphatides and of specific presynaptic proteins, e.g. synapsin-1,

syntaxin-3, post-synaptic protein PSD-95 and cytoskeleton protein F-actin (Wurtman et al. 2006). A number of studies have shown that omega-3 PUFA can modulate neurotransmission. Omega-3 PUFA supplementation can increase dopamine level in rat frontal cortex (Chalon et al. 1998). In contrast, chronic omega-3 PUFA deficiency significantly decreases dopamine storage vesicles (Zimmer et al. 2000) and dopamine levels (Delion et al. 1996). Furthermore, an omega-3 PUFA deficient diet also alters serotonergic transmission (Kodas et al. 2004) and cholinergic transmission in the rat hippocampus (Aid et al. 2003).

In SCI, few studies have discussed the effect of DHA on synaptic plasticity. In one study, dietary DHA acts synergistically with the effect of exercise on synaptic plasticity by increasing the mRNA levels of molecular markers of learning, i.e., BDNF, cAMP response element-binding protein (CREB), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and syntaxin-3 (Joseph et al. 2012). Several studies have shown that BDNF modulates synaptic plasticity (Bolton et al. 2000; Hariri et al. 2003). BDNF-related mechanisms are another possible way in which DHA may promote synaptic plasticity following SCI.

1.7.4.2 DHA promotes anatomical neuroplasticity

Recent studies demonstrate that DHA can increase both maximum neurite length and the total number of neurites in embryonic hippocampal cell cultures (Calderon et al. 2004), and even in mature neurons (Robson et al. 2010). Dietary supplementation with DHA following facial nerve injury appears to promote a pro-regenerative response

(Makwana et al. 2006). DHA treatment improves locomotor function and increases serotonin levels in lumbar spinal cord at 6 weeks following thoracic compression SCI (Ward et al. 2010). Topical DHA in combination with nerve growth factor can increase corneal nerve regeneration in rabbits (Esquenazi et al. 2005; Cortina et al. 2013). Such evidence supports the hypothesis that DHA has the ability to enhance axon growth.

The molecular mechanism underlying the promoting effect of DHA on anatomical neuroplasticity remains unclear. DHA can influence cell function through multiple mechanisms, ranging from modulation of membrane properties to regulation of signal transduction and gene expression. Omega-3 PUFAs are known to act as ligands for the RXR and PPARγ. For instance, nerve growth factor (NGF)-induced neuronal differentiation is mediated through activation of PPARγ in a TrkA-dependent manner (Fuenzalida et al. 2005). PPARγ activation also increases NGF and BDNF levels after SCI (Meng et al. 2011). Furthermore, a role of retinoid signaling (likely to be modulated by DHA) in the regeneration of sensory neurons in the spinal cord has also been demonstrated (Wong et al. 2006). Syntaxin-3 is a plasma membrane-bound protein, which is found in neuronal growth cones and is susceptible to the influence of DHA on membrane expansion and neurite outgrowth (Darios et al. 2006). A recent report showed that the level of syntaxin-3 is upregulated after DHA treatment in TBI (Wu et al. 2011). Thus, it is likely that DHA exerts its effect on neuroplasticity through multiple pathways.

1.8 Aims

DHA has promising potential in the treatment of SCI. The studies that follow are divided

into five parts

a) To characterize a cervical SCI model

b) To evaluate the neuroprotective effects of DHA in a cervical SCI animal model

c) To assess the effects of DHA on neuroplasticity in the cervical SCI animal model

d) To investigate the mechanism responsible for the effects of DHA on neuroplasticity

e) To examine the synergistic effect of DHA treatment and a rehabilitation program

2 Material and Methods

Experimental procedures in animals were performed in compliance with the Barts and the London, School of Medicine and Dentistry guidelines and were approved by the United Kingdom Home Office (Animal [Scientific Procedures] Act 1986). Surgery was carried out under anaesthesia and pain relief was provided appropriately during post-operative care.

2.1 Cervical hemisection SCI

To perform the injury to the spinal cord, adult female Sprague-Dawley rats (weight 225-249 g Charles River, Margate, UK) were deeply anaesthetized with 4% isoflurane (Merial, Essex, UK), as evidenced by lack of response to a nociceptive stimulus consisting of a paw pinch. The neck of the anaesthetized animals was shaved and disinfected with povidone iodine. A dorsal midline incision was made in the skin and superficial muscles of the neck region. Exposure of vertebral laminae C4, C5, and C6 was made through blunt dissection. The spinal process and vertebral laminae of C4 and C5 were removed without applying pressure on the underlying spinal cord, under microscope control. After opening the dura, the left side of the cervical spinal cord was cut at the middle of C4 and C5 with a microblade. The microblade was inserted into the spinal cord at the midline until the ventral surface of the vertebral column was touched. The knife was then pulled across the spinal cord until it came out the lateral side. This incision was repeated once and the completeness of the lesion was verified microscopically. Sham animals only received laminectomy to expose the spinal cord,

leaving the spinal cord undisturbed. After complete coagulation, muscles were sutured layer by layer and skin layers were closed with subcutaneous suture with 4-O absorbable suture (VICRYL Rapide Suture W9930). After surgery, animals were given buprenorphine subcutaneously (0.02 mg/kg; Reckitt Benckiser, UK) and then were placed in warm cages to recover from anesthesia.

2.2 Mouse pyramidotomy

The pyramidotomy was performed by Dr. Ping K Yip. Right pyramidotomy was performed on adult female CD1 mice (weight 25-29 g Charles River, Margate, UK) (n = 5-6 per group) using methods adapted from previous studies (Starkey et al. 2005; Yip et al. 2010). Mice were anaesthetised with a mixture of medetomidine (0.5 mg/kg) and ketamine (75 mg/kg) intraperitoneally. Surgical sterile procedures were adapted from previous studies (Thallmair et al. 1998; Starkey et al. 2005). Mice were prepared for surgery by shaving and disinfecting the ventral surface between the forelimbs and jaw. A ventral midline incision was made; the sterno-hyoid and sterno-thyroid muscles were lightly retracted. The trachea and oesophagus were carefully displaced. The ventrocaudal part of the bone was partially removed using forceps before the right pyramidal tract was incised with iridectomy scissors. The oesophagus, trachea and muscles were repositioned, and the skin sutured. Thirty minutes after pyramidotomy, animals received a tail vein injection of either vehicle (0.2% ethanol in saline) or DHA (500 nmol/kg) in a volume of 5 ml/kg. Post-operative care involved subcutaneous injection of analgesic (buprenorphine, 0.05 mg/kg) twice daily for 3 days following surgery.

2.3 Preparation and treatment of DHA for acute i.v. injection

2.3.1 Preparation of DHA for acute i.v. injection

1 M stock aliquots of free fatty acid (DHA; Sigma, Dorset, UK) were prepared in a polystyrene box under 100 % nitrogen and were made up in absolute ethanol. The 5 µl concentrated stock aliquots were then kept at -20℃ in light opaque, airtight glass containers (Agilent, Stockport, UK) to prevent oxidation. On the day of surgery, required concentration solutions of DHA were prepared in sterile saline (NaCl, 0.9% w/v) from the stock aliquot solutions of DHA.

2.3.2 Acute administration of DHA in animals following surgical intervention

Thirty minutes after hemisection in rats and pyramidotomy in mice, animals received a tail vein injection of either vehicle (0.2% ethanol in saline) or DHA (250 nmol/kg in rats, 500 nmol/kg in mice) in a volume of 5 ml/kg. The i.v. tail vein injections were carried out by Dr. Meirion Davies and the author was blinded to the treatment group. The animals received postoperative care including subcutaneous injection of analgesics (buprenorphine, 0.02 mg/kg body weight) and normal saline (2 ml) for 3 days following surgery.

2.4 Anterograde tracing

To evaluate whether the treatments had promoted axonal sprouting, we performed anterograde tracing of the intact CST as previously described (Yip et al. 2010). One week after the hemisection injury and 4 weeks after pyramidotomy in mice, animals were anesthetized again with 4% isoflurane. Animals were placed in a stereotaxic frame and seven burr holes were made into the skull ipsilateral to the hemisection and

contralateral to the pyramidotomy. In cervical hemisected rats, burr holes were made at the following coordinates defined as anteroposterior (AP), mediolateral (ML) related to bregma: (i) AP: -1.5 mm, ML: 2.5 mm; (ii) AP: -0.5 mm, ML: 3.5 mm (iii) AP: - 0.5 mm, ML: 2.5 mm; (iv) AP: + 0.5 mm, ML: 3.5 mm; (v) AP: + 1.0 mm, ML: 1.5 mm; and (vi) AP: 1.5 mm, ML: +2.5 mm; and (vii) AP: +2.0 mm, ML: 3.5 mm (Fig 2.1). In the pyramidotomy mice, the holes were made at the following coordinates: (i) AP: -1.0 mm, L: +0.5 mm; (ii) AP: -1.0 mm, L: +1.0 mm; (iii) AP: -0.5 mm, L: +1.0 mm; (iv) AP: -0.5 mm, L: +0.5 mm; (v) AP: 0 mm, L: +1.0 mm; (vi) AP: +1.0 mm, L: +1.0 mm; (vii) AP: +1.5 mm, L: +1.0 mm; (viii) AP: +2.0 mm, L: +1.0 mm. At each site, injections of biotinylated dextran amine (BDA; 10%; 10 000 MW, 1 μl/site for rat and 0.2 μl/site for mouse) were delivered using a glass micropipette attached to a Hamilton syringe via water-filled polyethene tubing. The micropipette was inserted below the skull surface (2 mm deep for rat and 1 mm for mouse) and BDA delivered at a rate of 0.2 μl/min. Animals were subsequently maintained for 2 weeks before tissue was collected for histology.

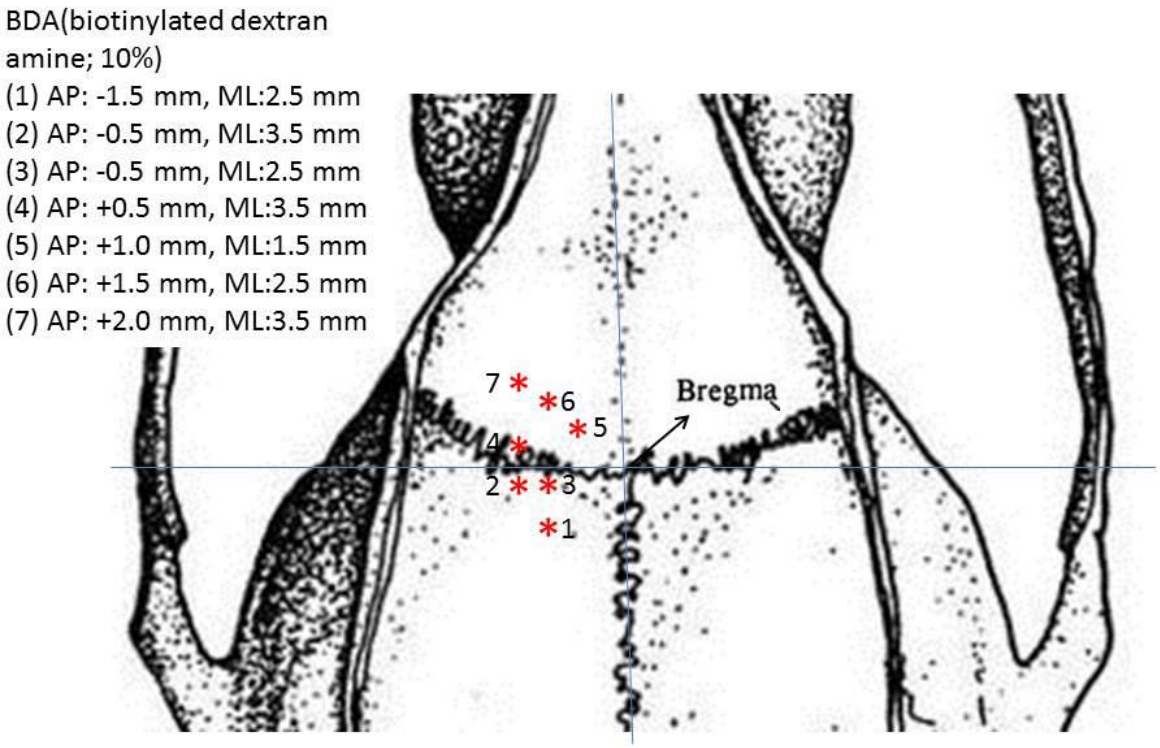

Figure 2-1 The coordinates for the neuronal tracer injection on the rat skull

The reference point is bregma, which is the anatomical point on the skull at which the coronal suture is intersected perpendicularly by the sagittal suture.

2.5 Tissue harvesting for immunohistochemical analysis

2.5.1 Perfusion, fixation and embedding of spinal cord and brain tissue

At the appropriate postsurgical interval, animals were deeply anesthetized with sodium phenobarbital (50 mg/kg, i.p.). Once the absence of corneal or forepaw reflexes was confirmed, a midline incision was performed along the chest and the animal was perfused through the ascending aorta with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The cervical spinal cord and brain was dissected out, postfixed in 4% paraformaldehyde for 2 hours, and cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight at 4°C. After embedding in OCT (Optimal Cutting Temperature compound) embedding medium (VWR, Lutterworth), tissue was frozen and stored at -80°C for subsequent processing for immunohistochemistry.

2.5.2 Cryosectioning of rat spinal cord

Serial horizontal cryostat spinal cord sections of 15 μm thickness were cut from C3 to C7, which segment was identified by the dorsal ganglion root. All sections were collected onto Superfrost glass microscope slides (VWR, Lutterworth, UK). The slides were then stored at -20°C until required for immunohistochemistry.

2.5.3 Cryosectioning of mice spinal cord

The cervical region (C3-7) of the pyramidotomy mice was sectioned (15 μm) in the transverse plane.

2.5.4 Cryosectioning of rat brain

To investigate the change in cortical neurons in the forelimb area of motor cortex after SCI, the rat brains were cut in a coronal manner from –0.5 to 5.5 mm rostral to bregma (Brus-Ramer et al. 2009).

2.6 Immunocytochemistry

Selected slides of brain and spinal cord sections were removed from -20°C and the chosen slides were washed with gentle agitation in PBS (3 x 5 min). Then, the sections were incubated in 10% normal donkey or goat serum for 30 mins followed by an overnight incubation with primary antibodies (Table 2.1).The next day, sections were washed three times (5 min each) in PBS before being incubated for 2 h in the appropriate secondary antibodies conjugated to Alexa Fluor 488 or 594 (1:1000). After another three 5 min washes in PBS, sections were then counterstained with the fluorescent nuclear dye bis-benzimide (Hoechst 33342; 0.2 mg/100 ml PBS; Sigma, UK) for 5 min to facilitate detection of cell nuclei or in NeuroTrace® 435/455 Blue Fluorescent Nissl (1:100, Life Technologies) to stain neuronal cell bodies. Slides were mounted in ProLong® Gold antifade reagent.

The tyramide signal amplification technique was carried out for the detection of BDA-labelled corticospinal tract fibers and PTEN immunostaining in the cortex. Sections were washed three times (5 min each) in PBS and then incubated with 0.3% hydrogen peroxide for 30 min. After further 3x5 min washes with PBS, the sections were incubated in avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories) for 30 min at room temperature. Following 3x5 min washes in PBS, sections were incubated with biotinel tyramide (1:75, NEN Life Sciences) for 10 min. After a further 3x5 min washes in PBS, the sections were incubated with extra-avidin FITC (1:400) for 2 hours before the sections were further immunostained with another antibody for double-labelling or washed, mounted, and cover slipped.

Table 2.1 Primary antibodies used

Primary antibody	Abbreviation	Concentration and species	Distributor	Specificity
Neuron-specific nuclear protein	NeuN	1:1000 Mouse	Chemicon UK	Mature neuronal cell bodies
Adenomatous polyposis coli tumor suppressor protein	APC	1:200 Mouse	Calbiochem	Oligodendrocyte cell bodies
Ionized calcium-binding adapter molecule	Iba-1	1:1000 Rabbit	Wako	Ca^{2+}-binding peptide produced by resting monocytes as well as activated microglial cells
Neurofilament H phosphorylated	SMI31	1:1000 Mouse	Sternberger,	Phophorylated epitope in the neurofilament heavy subunit
Serotonin	5-HT	1:3000 Rabbit	Immunostar	Serotonin coupled to bovine serum albumin (BSA) with paraformaldehyde
Anti-choline acetyltransferase	ChAT	1:100 Goat	Chemicon	Cholinergic neurons
Synaptophysin	SYP	1:1000 Rabbit	Cell Signaling Technology	Synaptic boutons

Visual system homeobox 2	Chx 10	1:100 Sheep	Abcam,	V2a group interneurons expression of the transcription factor Chx10
Phosphatase and tensin homologue deleted on chromosome ten	PTEN	1:250 Rabbit	Cell Signaling Technology	Endogenous levels of total PTEN protein in cells
Protein kinase C gamma	PKCγ	1:500 Rabbit	Santa Cruz Biotechnology,	Intracellular signaling kinase found in axons of the corticospinal tract

2.7 In situ hybridization

In situ hybridization (ISH) was performed on brain cortex sections as described previously (Lopez-Ramirez et al. 2014) except for minor changes. Sections were first washed in PBS for 5 minutes for 3 times prior to proteinase K treatment 2 μg/ml at 37^{o}C for 10 minutes, performed in water bath. Sections were then fixed again for 5 min in 4% PFA and dehydrated in 70%, 96% and 99.9% ethanol twice, for one minute each time. Double digoxigenin–labeled miRcuryLNA probe miR-21 oligonucleotide (5 nM; Exiqon, Vedbaek, Denmark) was hybridized with the sections for one hour at 53°C. After hybridization, the sections were washed in a series of 5 minutes saline-sodium citrate (SSC) washes at 53°C, consisting of 3 washes with 5x SSC, 2 times in 1x SSC 3 washes in 0.2x SSC, and incubated for 15 minutes in a blocking solution (0.05% Tween and 1% sheep serum) prior to incubation overnight in sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche), diluted to a concentration of 1:800 in dilution buffer (0.05% Tween,1% sheep serum and 1% bovine serum albumin). Next, the sections were washed with TBST 3 times, 5 min each, before incubation for 5 minutes twice in alkaline development buffer (100 mM Tris HCl, PH 9.5; 100 mM NaCl; 50 mM MgCl2; and 0.1% Tween-20), and then the sections were incubated with NBT/BCIP (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (1%; Roche) and levamisole (0.5%; Vector Laboratories, Peterborough, UK), at 30°C overnight. Finally, sections were washed in KTBST (50 mM Tris Hcl PH7.5, 150 mM NaCl, 20mM KCl, 0.5% Tween-20) for 5 minutes twice to stop the reaction, then washed with water one minute twice. The slides were dehydrated at room temperature overnight, then sections were mounted and coverslipped with mounting medium.

2.8 Western blotting

Spinal cord tissue was separated into several segments, rostral and caudal to the lesion site. Each sample was homogenized in ice-cold lysis buffer (20 mM HEPE PH 7.4, 100 mM NaCl, 100 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 1X protein inhibitor, 1% nonidet P-40). Lysates were left under rotation for 2 h at 4℃ before centrifugation (15000 *g*, 15 min, 4℃) and collection of the supernatant. The protein concentration of lysates was estimated using a colorimetric protein assay according to the manufacturer's instructions (BCA protein assay (Pierce UK). 10 μg of total protein from each sample was electrophoresed in loading buffer (60 mM TrisCl PH 6.8, 2% SDS, 1% beta-mercaptoethanol, 5.6% glycerol, 0.02% bromophenol blue) across a 4.5% acrylamide stacking gel (20 min at 90 V) and 12% acrylamide resolution gel (90 min at 110 V). Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer method (60 min, 120 V). Membranes were then blocked in skimmed milk (5%, 30 min) prior to incubation overnight with a primary antibody: rabbit synaptophysin (1:1000; cell signaling, Technology) and mouse anti-β –III tubulin (1:1000; Promega). Membranes were washed and then incubated with secondary antibodies (donkey anti- rabbit IRDye 800, goat anti-mouse IRDye 680, 2 hr; Licor Biosciences) and visualized using the Odyssey infrared imagining system (LiCor Biosciences). Integrated band intensities for synaptophysin and β–III tubulin were quantified for each sample using Image J software. Densitometric values for synaptophysin were normalized against β –III tubulin for each sample.

2.9 Primary cell culture with DHA and sodium selenite treatment

Adult female Sprague-Dawley rats (225–250 g) were killed according to UK Home Office regulations. The dorsal root ganglia (DRGs) were dissected and transferred to Ham's F12 medium (Gibco). DRGs were desheathed and trimmed, digested in 0.125% collagenase (Sigma) at 37°C for 2 h, and mechanically dissected by trituration with a P1000 Gilson pipette in 1 ml of modified Bottenstein and Sato's culture medium (BS) in Ham's F12. The cell suspension was centrifuged at 800 g for 6 min through a cushion of 15% bovine serum albumin (BSA, Sigma). The dissociated neurons were diluted in modified BS culture medium to approximately 1,600 cells per ml. Cells (500 per well) were plated in 4-well plates (Labtek) that were precoated with laminin (0.1 μg/ml Sigma). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

For experiments, the next day (1 day *in vitro* (DIV)), DHA (1-10 μM) or control media (BS media with 0.008% ethanol) were added to the DRG cultures. At 2 DIV, sodium selenite (10 μM) was added to the cultures to induce PTEN expression (Berggren et al. 2009; Luo et al. 2013). At DIV3, neurons were fixed for 20 min in 4% paraformaldehyde and permeabilized with methanol at –20℃ for 5 min, washed with phosphate-buffered saline (PBS) and incubated at room temperature for 2 h with a combination of mouse β-III tubulin (1:1,000) with rabbit PTEN (1:200, Cell Signaling Technology). Secondary antibody staining was performed with a mixture of Alexa Fluor 488 (1:1,000, Molecular Probes) and Alexa Fluor 594 (1:1,000, Molecular Probes) at room temperature for 45 mins. After PBS washes, cells were mounted with FluorSave reagent (Calbiochem) and observed under a fluorescence microscope.

2.10 Image capture and analysis

2.10.1 Image capture and data analysis for the DHA study in rats

All analyses were performed blind. Regions from at least 3 sections per animal were used for quantification or measured and data expressed as means ± S.E.M.

2.10.1.1 NeuN positive cells and APC positive cells

To determine the number of spinal cord neurons and oligodendrocytes, sections were viewed on a Leica epifluorescence microscope using tetramethylrhodamine isothiocyanate (Y3) or fluorescein isothiocyanate filter blocks and photographed at 20X magnification. All NeuN and APC immunoreactive cells were also visualized using the filter for Hoechst, which showed labelled nuclei. To maintain consistency with previous work, measuring frames at specific locations were used (Fig 2.2). Quantitative analysis of the number of Hoechst-labelled NeuN cells was conducted by capturing an image of NeuN-labeling and Hoechst labeling at various levels rostral and caudal to the lesion site. All captured cells were then counted in a 500 μm X 500 μm measuring frame by using the Image J analysis programme (Image J 1.46u, National Institute of Health, USA). A similar analysis was conducted for APC-labelled cells, with the exception that areas chosen for APC were in the ventral white matter.

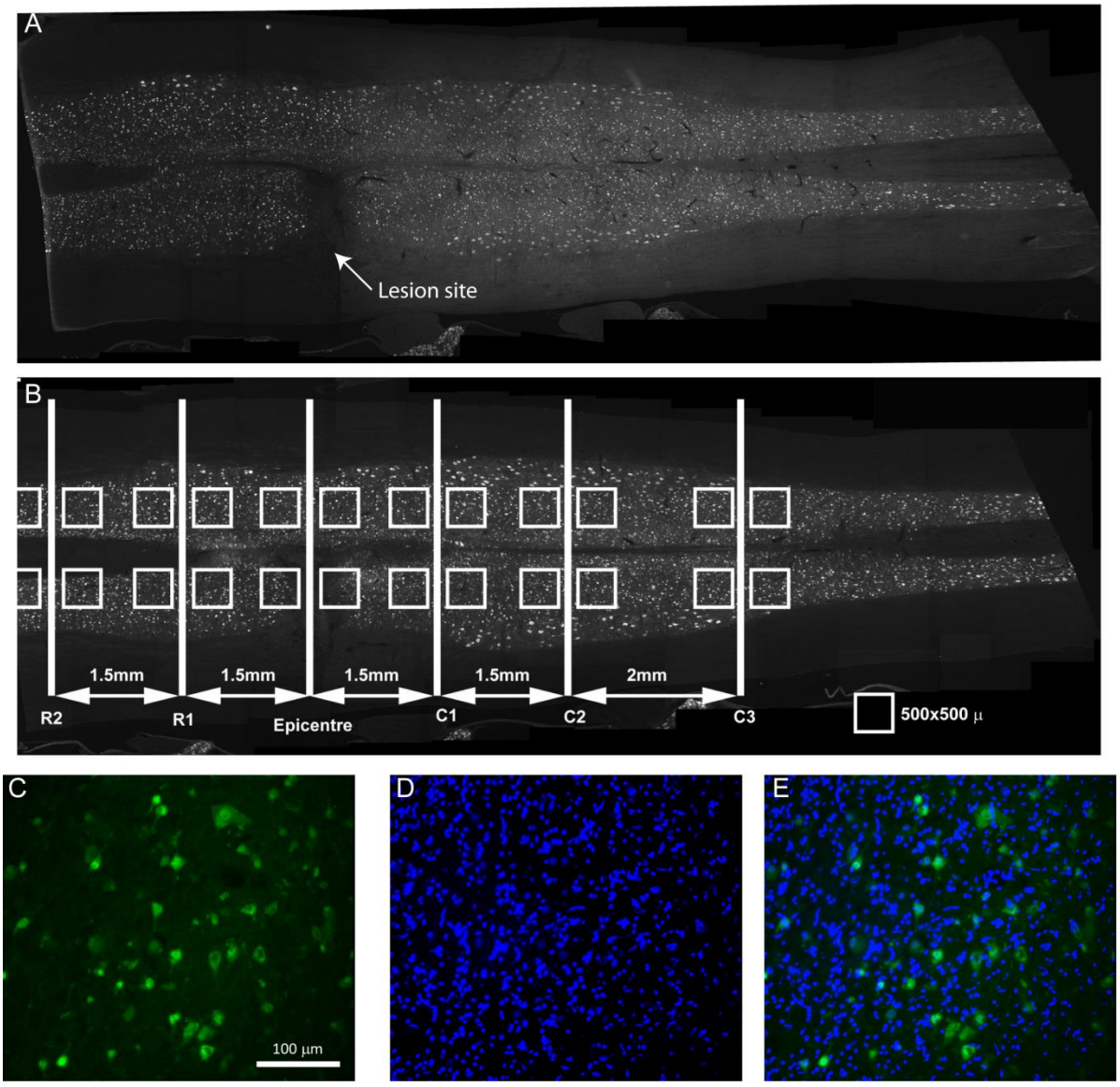

Figure 2-2 The template of neuronal cell counting

(A) The presented image is a montage image of rat spinal cord with NeuN staining. (B) The diagram shows a horizontal section through the injury epicenter and the various measuring frames caudal and rostral to the lesion site used to analyse different biochemical markers. (C) NeuN immunoreactive neuronal cells are shown as seen in one frame. (D) The section also was double stained with Hoechst, labelling nuclei. (E) The number of Hoechst-labelled NeuN cells was assessed.

2.10.1.2 SMI31 neurofilament, serotonin fibers, pre-synaptic protein (synaptophysin)

High power images (20X objective) were taken of specific areas. In the Image J analysis program, these images were converted to binary overlay and were counted automatically using the "analyse particles" programme. The programme summarized the percentage area of 500 μm x 500 μm squares that are occupied by immunostaining structures.

2.10.2 Image capture and data analysis for the DHA study in mice

For the mouse pyramidotomy study, tissue sections were viewed on a Leica epifluroscence microscope using a 20x objective. The quantitative analysis of Chx10 positive interneurons in all groups in spinal cord tissue was conducted by counting all labelled cells within the field of view in the areas of the intermediate column and ventral horn. In order to correlate with functional recovery, the number of Chx10 positive interneurons contacted by sprouting fibres was also identified and recorded. The histological assessment of BDA-labelled axons crossing the midline was done in the cervical spinal cord by a BSc student, Mr. Jae Won Lee under Dr. Ping K. Yip supervision.

2.10.3 Image capture and data analysis for the DHA study in primary cell culture

During the course of this study, slides were viewed and images captured using a Leica epifluorescence microscope (Wetzlar, Germany). Ten images from each cover slip were captured at 20X magnification using the Image J analysis program in the regions shown (Fig. 2.2). This quantification format enables a representation of the whole cover slip to be captured and analysed. The expression level of PTEN, the length of the neurites and

the number of neurites of individual DRG cells was measured by Image J software. Cultures for each group were performed in duplicate, and for each well 200-400 neurons were quantified. Cultures were repeated four times.

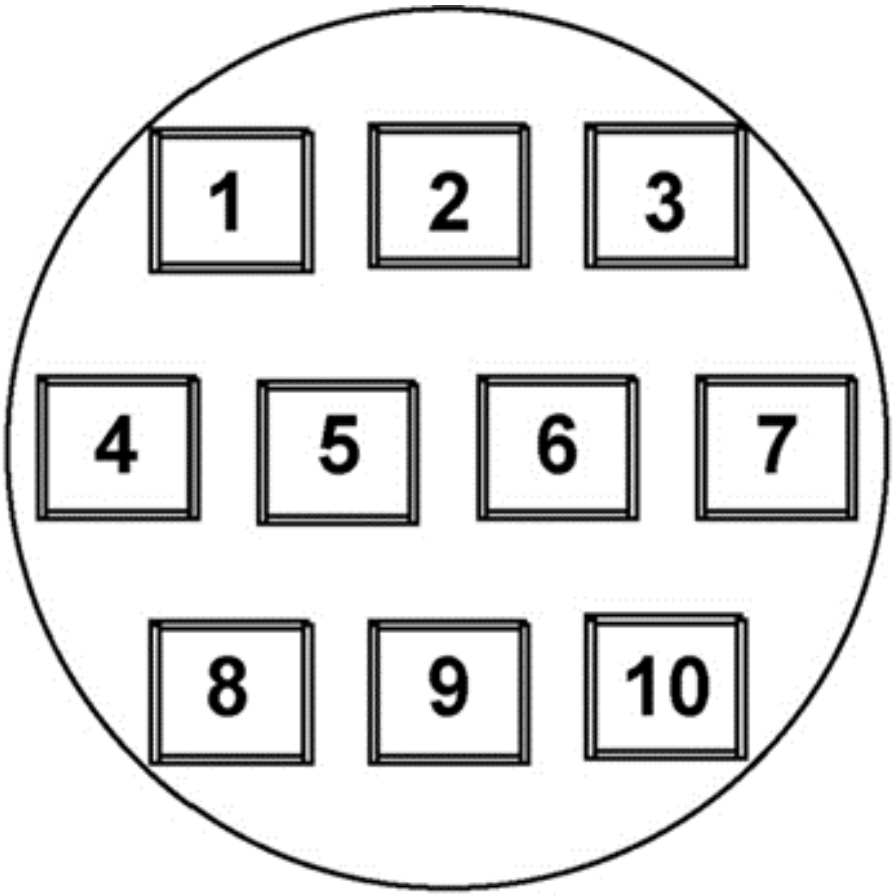

Figure 2-3 Capture and analysis of cell immunostaining intensity

Ten images, denoted by numbers, were captured in the indicated regions to provide a representation of the cellular coverage of the whole cover slip.

2.11 Behavioural studies

2.11.1 Open field locomotion

Impairments in hindlimb and forelimb locomotor skills after cervical SCI were evaluated using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale (Basso et al. 1995) and the forelimb locomotor scale (FLS) (Cao et al. 2008). Animals were allowed to acclimatize preoperatively to the testing environment in an open field, which was a circular plastic enclosure. Forelimb and hindlimb function were evaluated in the open field, which measured approximately 3 feet in diameter, and rats were observed for 4 minute intervals. The open field test was conducted 1 day after surgery, then every day for 2 weeks. After 2 weeks, the test was performed every other day for another week.

2.11.2 Staircase test

The Montoya staircase test (Montoya et al. 1991) is a well-established measure of forepaw motor function in rats and has been demonstrated to be reproducible and sensitive to motor impairments. In our study, animals were trained on the Montoya staircase test daily for 14 days prior to SCI. We then performed the test every day for one week after injury and every other day from one week to three weeks post-injury. Two parameters were assessed in this task. One is the number of food pellets retrieved and eaten by animals (skilled limb control) and another one is the number of food pellets displaced or retrieved (general limb control). One food pellet was placed per step, and the number displaced and the number eaten was recorded.

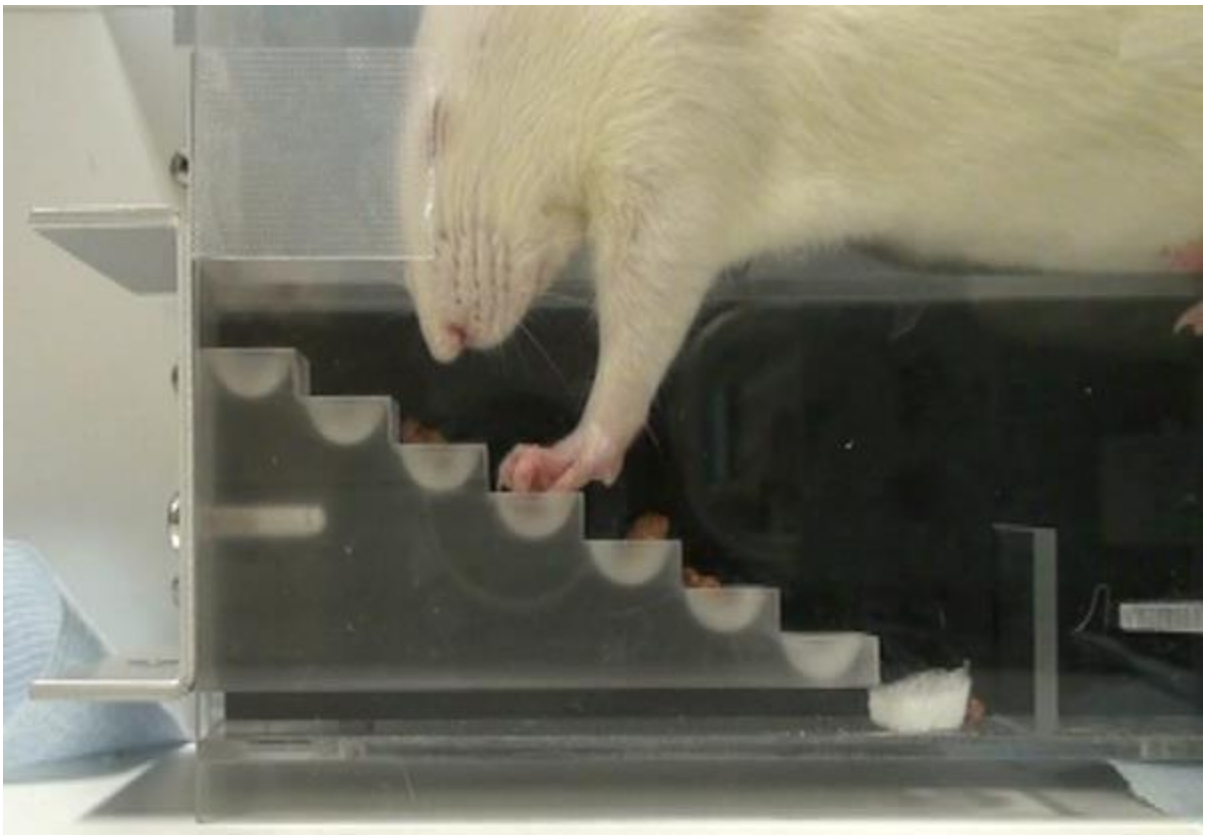

Figure 2-4 Forepaw reaching following cervical hemisection injury

A side view of a rat in the staircase apparatus. The left forearm is stretching to reach a food pellet in the fourth stair.

2.11.3 Grid exploration test

The grid-walk test focuses on voluntary aspects of limb movements (Metz et al. 2000). Deficits in descending motor control can be examined by assessing the ability of a rat to precisely control and place its hind paws on a horizontal, ladder-like grid. The animals are placed on the grid for 3 min and allowed to walk freely across the space. The wire mesh is about 40 cm×60 cm containing 5 cm×5 cm mesh, raised 20 cm high. A video camera was located below the grid apparatus, with an angle of about 20-40 degrees. Behavior on the grid was recorded on video film. One misplacement was counted when

the limb protruded entirely through the grid and extended below the wire surface. All animals were required to receive the grid exploration test 3 times before surgery, to gain the baseline data and get used to the mesh grid. The post-surgery testing was performed weekly until the end of the study.

Figure 2-5 forepaw slip demonstration during grip exploration test

The picture demonstrates the left forepaw slip during the grid exploration test in rats following cervical hemisection.

2.11.4 Footprint test

To assess stepping patterns of forelimbs and hindlimbs after injury, animals were required to run along a paper-lined runway (120 cm long, 7 cm wide) to obtain a food treat in a darkened box at the end of the runway. The plantar surfaces of forelimbs and hindlimbs were brushed with red and black nontoxic ink. Footprint analysis was performed on the day prior to surgery and at postoperative days 2, 7, 14, 21.

Fig 2.6 Footprint test.

The photograph demonstrates the rat running along the paper-lined runway to the darkened box.

2.11.5 Task-specific rehabilitation

Staircase training has been shown to improve food pellet grasping after cervical spinal cord (Garcia-Alias et al. 2009) and brain ischaemia lesions (Maldonado et al. 2008). Recovery of reaching and pellet retrieval is associated with plasticity of the corticospinal and rubrospinal tracts and motor cortex, and requires integration of segmental, intersegmental, and supraspinal input to propriospinal interneurons and motoneurons over many spinal levels (Whishaw et al. 1998; Stackhouse et al. 2008).

For 2 weeks before injury, animals were trained to grasp and eat food pellets from the Montoya-type staircase device, the same as the one used for staircase rehabilitation. 2 days after cervical spinal cord hemisection, the animals were started on task-specific rehabilitation. One food pellet was put on the right side wells (non-lesion side), and a

variety of food pellets were put on the left side so the animals were encouraged to grasp the food pellets with their injured forepaw. The training was performed for 30 min twice daily.

2.12 Statistical analysis

The behavioural assessment and histological analysis were performed blind. All statistical analyses were performed using GraphPad Prism Version 6 (Gradpad software Inc., San Diego, CA, USA). The data were presented as means and standard error of the means. One-way or two-way repeated measures ANOVA was used to compare experimental groups, with Tukey's post-hoc comparisons. Differences were considered significant when $p < 0.05$.

3 Characterization of cervical hemisection SCI

3.1 Introduction

The majority of SCI occurs at cervical level (Jackson et al. 2004) and most of these injuries are 'incomplete' (Raineteau et al. 2001). To establish a clinically relevant cervical spinal cord animal model, hemisection of the cervical spinal cord was applied to our animals. By disrupting both ascending and descending pathways on one side, spinal hemisection provides an interesting model to study plastic changes that may differentially affect the somatosensory and motor cortical representations, as well as their potential implications for behavioural recovery.

3.1.1 Models of cervical SCI

Patrick D Wall in a personal communication to the International Spinal Research Trust (Ramer et al. 2000), has stated four characteristics that are required for an optimal model of SCI. These are as follows:

1. The extent of the lesion should be precisely defined. If there is doubt about the extent of a lesion or whether axons have been spared, then the interpretation of regeneration can be misleading.
2. A histological method should be available to detect the growth of axons through the lesion. In order to visualize specific pathways and allow the comparison of different pathways following treatment, anterograde and retrograde tracers and biomarkers should be applied to the animal models.
3. An electrophysiological method should be available capable of detecting functional

synaptic transmission beyond the lesion. Synaptic connection examination will be essential to determine the function of regenerating axons.

4. A behavioral measure should be available, capable of detecting restoration of known circuits. The behavioral tests are necessary to assess whether functional recovery has been achieved.

In addition to these characteristics, the animal model should reproduce as closely as possible the anatomical and physiological changes that occur in human SCI.

The first significant attempt to produce a clinically relevant model of SCI was that of Allen (Allen 1911). In this paradigm, a defined weight is dropped from a known height onto the dorsal surface of the exposed thoracic spinal cord. However, the first cervical SCI was described in 1992 (Anderson et al. 1992), and established the feasibility of a C4—C5 spinal cord contusion injury model in the rat. The study was focused on the degree and persistence of forelimb behavioral deficits following cervical spinal cord contusion injury. In 1993, Schrimsher and Reier (Schrimsher et al. 1993) developed a partial section of the spinal cord, to establish the role of various long-tract spinal pathways in forelimb motor control. Recently, there has been an increasing interest in the development and characterization of rodent cervical SCI (Dai et al. 2009; Martinez et al. 2009; Dunham et al. 2010), largely due to the high clinical relevance and great potential to evaluate the neuroprotection or neuroplasticity that involved both gray and white matter following injury. Compared with the often used mid-thoracic SCI, return of segmental function is easier to detect and analyze in cervical injury models (Anderson et al. 2007). In addition, cervical spinal injury models offer another advantage,

evaluating the function of paw and digits that are directly controlled by cortical input (Whishaw, Pellis et al. 1993).

Many studies use the thoracic SCI model and evaluate locomotion, which is one of the most crucial neurological functions. However, most locomotor function is regulated by central pattern generating locomotor networks in the lumbar spinal cord that are triggered by activity in the reticulospinal tract (Rossignol et al. 1996). In human beings, supraspinal input plays an essential role in locomotor control, in particular from the CST for fine motion. Animal models which can assess conduction and control from supraspinal neurons may therefore be more clinically relevant. A recent questionnaire was raised to survey the opinion of clinical and scientific members of the SCI community. Ninety-four percent voted that the cervical models were more relevant than thoracic models, and only 6% voted that the two were equally relevant, after some discussion concerning a therapy being administered to cervical SCI patients (Kwon et al. 2011).

3.1.2 Different types of cervical SCI animal models

In recent years, considerable concern has arisen over the application of cervical SCI animal models in SCI research. Table 1 lists published rodent cervical SCI studies. Three common approaches (section-based injury, contusion injury, and clip compression) are used as experimental models that aim to mimic the type of cervical SCI that is seen clinically. The most common model is the section-based injury, in which specific axon pathways or specific areas of the cord are damaged in order to study the

response to injury of that particular region. Some studies have employed the lateral hemisection (half the cord) model, to investigate the effects of various treatments in promoting axon regeneration and neuroplasticity (References 3,6,9-12, 15, 18-19, 23, 26-27, 29, 33-34, 36-37, 40, 44-45,47 in Table 1). The second most common approach is to use a weight drop method or computed impaction system, which models contusion injury (References 1, 4, 7, 13, 16, 20, 24, 28, 32, 37-38, 41-42, 47 in Table 1).

Table 3.1 Rodent cervical SCI animal models

	Source of Paper	Injury level	Species	Injury type	Purpose of study
1	(Schrimsher et al. 1992)	C4/5	Rat	Contusion	Characterize animal model
2	(Schrimsher et al. 1993)	C4	Rat Female	Partial section	Characterize animal model
3	(Ye et al. 1997)	C2/3	Rat	Lateral hemisection	Neuroregeneration agent evaluation (neurotrophic factor)
4	(el-Bohy et al. 1998)	C2 & C4/C5	Rat Female	Contusion	Respiratory function following SCI
5	(Ballermann et al. 2001)	C1	Rat Female	Partial section	Sensory assessment following SCI
6	(Golder et al. 2001)	C2	Rat Female	Lateral hemisection	Respiratory function following SCI
7	(Soblosky et al. 2001)	C4-5	Rat Female	Contusion	Characterize animal model
8	(Casha et al. 2001)	C7	Rat Female	Clip compression	Pathological change following SCI
9	(Webb et al. 2002)	C3	Rat Female	Lateral hemisection	Behavioral compensation following SCI
10	(Anderson 2004)	C5-T1	Mice Female	Lateral hemisection	Characterize animal model
11	(Vinit et al. 2005)	C2	Rat Female	Lateral hemisection	Pathological change following SCI
12	(Anderson et al. 2005)	C5	Rat Female	Lateral hemisection	Characterize animal model
13	(Pearse et al. 2005)	C5	Rat Female	Contusion	Characterize animal model
14	(Onifer et al. 2005)	C4	Rat	Partial section	Characterize animal model
15	(Fujiki et al. 2005)	C2	Rat Female	Lateral hemisection	Neuronal pathway reorganization
16	(Gensel et al. 2006)	C5	Rat Female	Contusion	Characterize animal model
17	(Massey et al.	C6/7	Rat	Partial section	Neuroplasticity-promoting agents

	2006)		Male		evaluation (ChABC)
18	(Vinit et al. 2006)	C2/3	Rat Male	Lateral hemisection	Respiratory function following SCI
19	(Anderson et al. 2007)	C5	Rat Female	Lateral hemisection	Characterize animal model
20	(Onifer et al. 2007)	C5/6	Rat	Bilateral contusion	Electrophysiology change following SCI
21	(Cao et al. 2008)	C4	Rat	Partial section	Neuroplasticity-promoting agents evaluation (NEP1-40)
22	(Stackhouse et al. 2008)	C3-4	Rat Female	Partial section	Neurological deficit assessment following SCI
23	(Ying et al. 2008)	C4	Rat male	Lateral hemisection	Neuroplasticity-promoting therapy evaluation (BDNF + exercise)
24	(Anderson et al. 2009)	C5-8	Rat Female	Bilateral contusion	Characterize animal model
25	(Garcia-Alias et al. 2009)	C4	Rat	Partial section	Neuroplasticity-promoting therapy evaluation (ChABC + Rehabilitative training)
26	(Martinez et al. 2009)	C4	Rat Male	Lateral hemisection	Characterize animal model
27	(Martinez et al. 2009)	C4-5	Rat Male	Lateral hemisection	Activity based therapy evaluation
28	(Beaumont et al. 2009)	C4/5	Rat Female	Bilateral contusion	Neuroprotective agent evaluation (Rolipram)
29	(Strong et al. 2009)	C4	Rat Female	Lateral hemisection	Forelimb control following SCI
30	(Krajacic et al. 2010)	C2/3	Rat Female	Partial section	Activity based therapy evaluation
31	(Harel et al. 2010)	C3/4	Mice	Partial section	Neuroplasticity-promoting therapy (Nogo deletion)
32	(Dunham et al. 2010)	C5	Rat Male	Unilateral Contusion	Characterize animal model
33	(Martinez et al. 2010)	C4/5	Rat Male	Lateral hemisection	Cortical reorganization following SCI

34	(White et al. 2010)	C2	Rat Female	Lateral hemisection	Respiratory outcome following cell transplantation
35	(Lee et al. 2010)	C5	Rat	Unilateral contusion	Neuroprotection agent evaluation (simvastatin and minocycline)
35	(Filli et al. 2011)	C4/5	Rat Female	Lateral hemisection	Neuronal pathway reorganization
36	(Dai et al. 2011)	C4/5	Rat Female	Lateral hemisection	Activity based therapy evaluation
37	(Lane et al. 2011)	C3/4	Rat Female	Bilateral Contusion	Respiratory function following SCI
38	2012 Simard(Simard et al. 2012)	C7	Rat Female	Unilateral Contusion	Neuroprotection agent evaluation (glibenclamide and riluzole)
39	(Khaing et al. 2012)	C3-4	Rat female	Lateral hemisection	Forelimb function assessment following SCI
40	(Nguyen et al. 2012)	C7-T1	Rat Female	clip compression	Neuroprotection agent evaluation (IgG)
41	(Walker et al. 2012)	C5	Rat female	Unilateral contusion	Neuroprotection agent evaluation (Bisperoxovanadium)
42	(Cote et al. 2012)	C5	Rat Female	Unilateral Contusion	Neuronal pathway reorganization following SCI
43	(Lopez-Dolado et al. 2013)	C6	Rat Male	Lateral hemisection	Neuroanatomical change following SCI
44	(Fouad et al. 2013)	C5/6	Rat Female	Lateral hemisection	Neuroplasticity-promoting therapy (BDNF,NT3 expression cell graft)
45	(Weishaupt et al. 2013)	C3/4	Rat Female	Partial section	Neuroplasticity-promoting therapy (BDNF, NT3, + Rehabilitative training)
46	(Kolar et al. 2014)	C3/4	Rat Female	Lateral hemisection	Stem cell therapy evaluation (adipose-derived stem cells)
47	(Singh et al. 2014)	C5	Rat	Unilateral contusion	Forelimb function assessment following SCI

3.1.3 The choice of the level of cervical lesion

In an animal model of cervical SCI, it is important to define a rationale for the choice of a particular injury level. For example, for a lesion at the C4-5 level of the cervical spinal cord:

1. C4 and C5 are the most common levels for cervical injuries in patients.
2. The cervical cord innervates the deltoids (C4), biceps (C4-5), wrist extensors (C6), triceps (C7), wrist flexors (C8), and hand muscles (C8-T1). In interpreting the consequences of cervical injury, it is important to note that lesions at C5 are upper motor neuron lesions with respect to the muscles of the forearm (McKenna et al. 2000).
3. The animal model can be used to mimic the dysfunction of upper limbs in patients after cervical SCI.
4. The C4 (the phrenic nerve system) also innervates the diaphragm. Cervical lesions above the C4-5 level may affect respiration, which would introduce further complications.

3.2 Aims

Building an effective experimental animal model is important to understand the pathophysiology after SCI and to develop and validate further therapeutic strategies. Our group has successfully studied rodent compression and hemisection models of thoracic SCI (Huang et al. 2007; Huang et al. 2007; Lim et al. 2013). These have allowed us to study in detail the pathogenesis of primary and secondary SCI, and to investigate the effects of various therapeutic agents. Our hypothesis is that a cervical

hemisection animal model can increase our basic understanding of mechanisms involved in cervical SCI and recovery. The present study was aimed at characterizing a reliable and reproducible method of producing experimental cervical hemisection SCI in the rat and investigating the effects of a cervical spinal hemisection on sensorimotor performance in relation to the histological changes in the cervical spinal cord. In addition, this model forms the basis for further studies in this thesis, evaluating the neuroprotective and plasticity-promoting effects of DHA.

3.3 Results

The work presented here demonstrates the development of a rat cervical hemisection SCI by behavioural observation, assessed by locomotor score (FLS and BBB), Montoya staircase test, grid exploration test, and foot print analysis. Three weeks after injury, we employed immunohistochemical techniques to examine the histological changes following cervical hemisection.

3.3.1 Effect of cervical hemisection SCI on neurons

Three weeks after surgery, we used mouse neuron specific nuclear protein (NeuN) to examine neuronal survival. In laminectomy controls, more NeuN-labelled neurons were present in the spinal cord than in the hemisection animals (Fig 3.1.A,B). In the cervical spinal cord hemisection group, a loss of NeuN immunoreactivity was noted in the vicinity of the spinal cord lesion site (Fig 3.1.C,D). Quantitative analysis confirmed that significantly lower numbers of NeuN-labelled cells were found in spinal cord tissue 3 weeks after left cervical spinal cord hemisection injury than in sham operation controls. In the sham animal group, the number of neuronal cells per 0.25 mm^2 in cervical spinal cord 250 μm caudal (74.5±5.8 v.s 18.8±6.9, $p<0.05$) and rostral (68±9.8 v.s 8.5±3.5, $p<0.05$) to the lesion site is significant higher than the hemisection group (Fig 3.1.E).

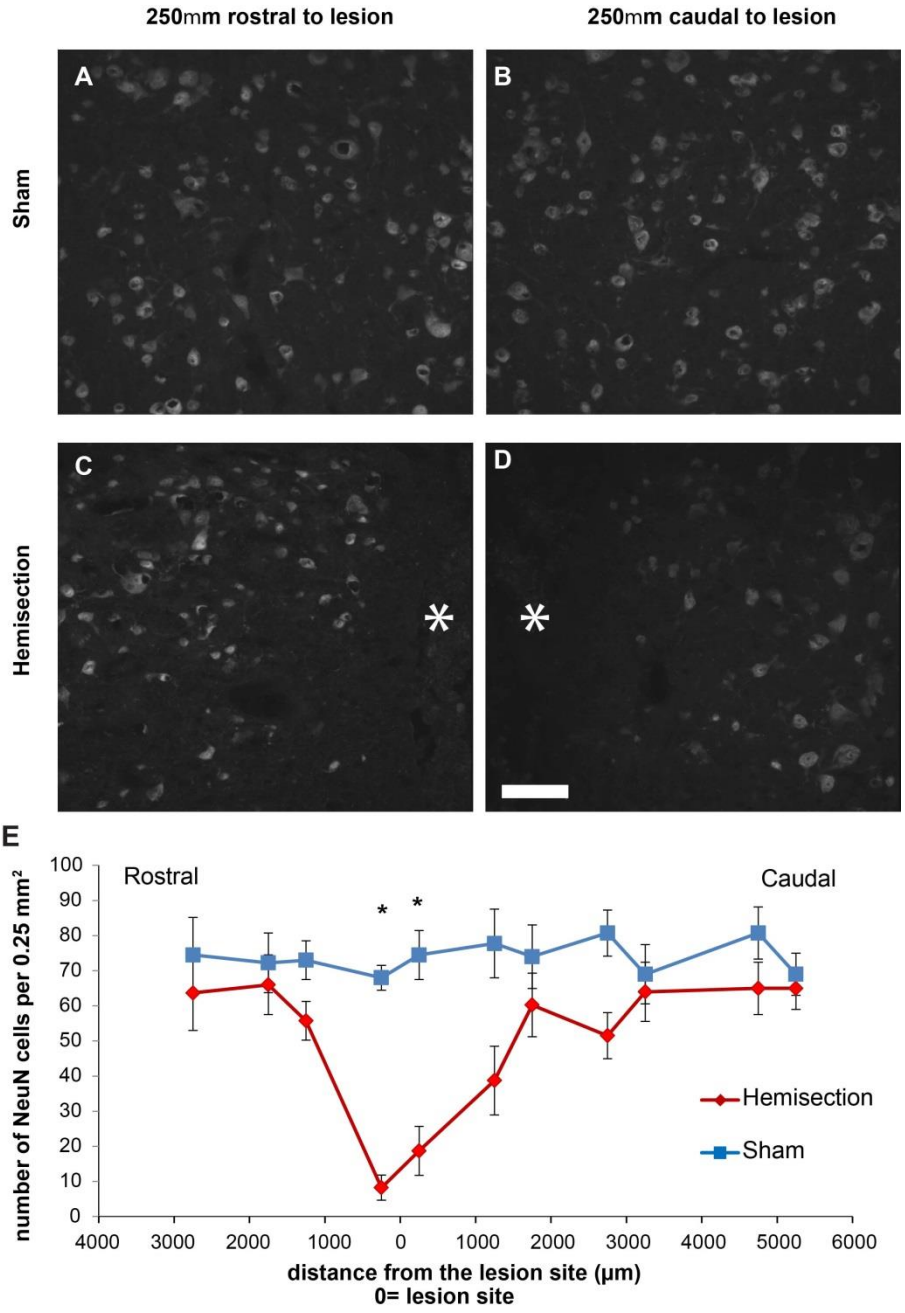

Figure 3-1 NeuN staining and quantification of neuronal cells in cervical SCI and sham operation animals

The representative images in A-D were taken through the spinal cord rostral (A,C) and caudal (B,D) to the lesion. The images (A,B) from the sham surgery group revealed numerous NeuN-labelled cells. The number of NeuN-labelled cells was diminished in the spinal cord 250 μm rostral (C) and 250 μm caudal (D) to the lesion site in the hemisection group. Stars denote lesion site. Quantitative analysis of NeuN immunostaining (E) revealed that there was a significant loss of NeuN in the epicenter region (*P<0.05). Results represent mean ±SEM; n=4 animals in each group. Scale bar =100 μm.

3.3.2 Effect of cervical hemisection SCI on oligodendrocytes

Mouse adenomatous polyposis coli (APC) tumor suppression protein was used to examine the survival of oligodendrocytes following SCI. In the spinal cord of control rats, APC-labelled oligodendrocytes were present throughout the white matter in the spinal cord. (Fig 3.2.A,B). In contrast to sham operation animals, there was a dramatic loss of APC-labelled cells in the cervical hemisection group ((Fig 3.2.C,D). The analysis of APC labelling showed a similar effect to that for NeuN labelling. Quantitative analysis confirmed a significant decrease in APC-labelled oligodendrocytes in the epicenter region as compared to the sham surgery group (49.5 ± 6.5 v.s 20.5 ± 5.5 per 0.25 mm^2, $p<0.05$) (Fig 3.2.E).

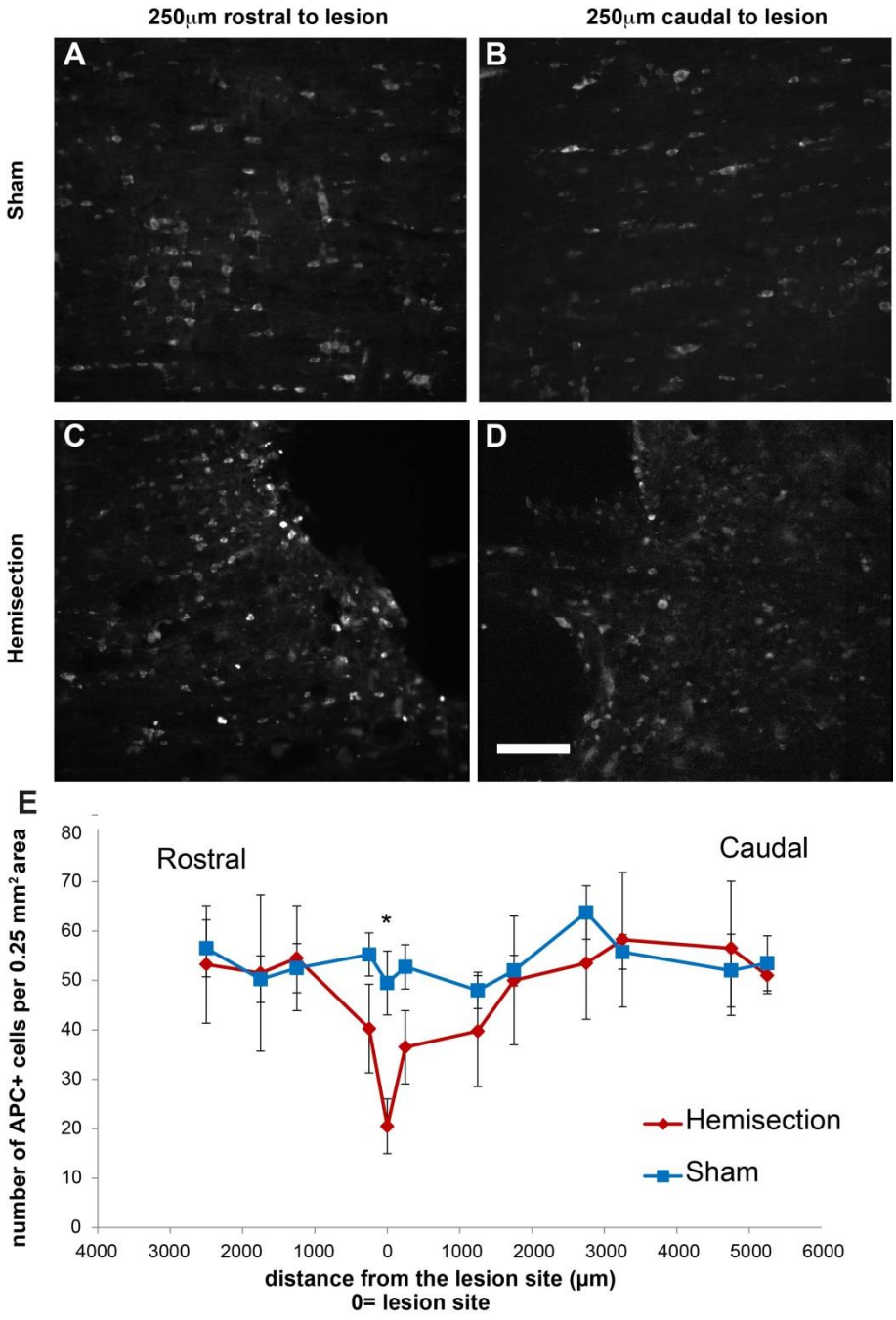

Figure 3-2 APC staining and quantification of oligodendrocytes in cervical SCI and sham operation animals

Representative immunohistochemical labelling of APC oligodendrocytes, 250 μm rostral (A, C) and 250 μm caudal (B, D) to the lesion site. In the sham operation group, APC immunoreactive cells are present throughout the white mater of the spinal cord (A, B). After cervical hemisection (C, D), a loss of APC labelled cells was seen in the epicentre. Quantification revealed a significant loss of APC immunoreactive cells in the epicentre white matter region (* $p<0.05$). Results represent mean ± SEM; n=4 animals in each group. Scale bar =100 μm.

3.3.3 Effect of cervical hemisection SCI on microglia/macrophages

Rabbit ionized calcium-binding adapter molecule-1(Iba-1) was used to evaluate the changes in microglia following SCI. Immunohistochemical examination showed that Iba-1 protein is expressed by quiescent as well as activated microglia (Ito et al. 1998). In the control group, the resting microglia were characteristically small cells with long and ramified processes (Fig 3.3.A, A'). In the hemisection group, microglia were transformed into an activated form and appeared as hypertrophied and bushy cells (Fig 3.3 C, C'). The data showed heavily stained microglia in the epicenter area compared to the shame operated rats (7.2 ± 2.1 vs. 1.05 ± 0.2, $p<0.05$), and this increase was apparent as far as 2-3 mm from the lesion site (Fig 3.3E).

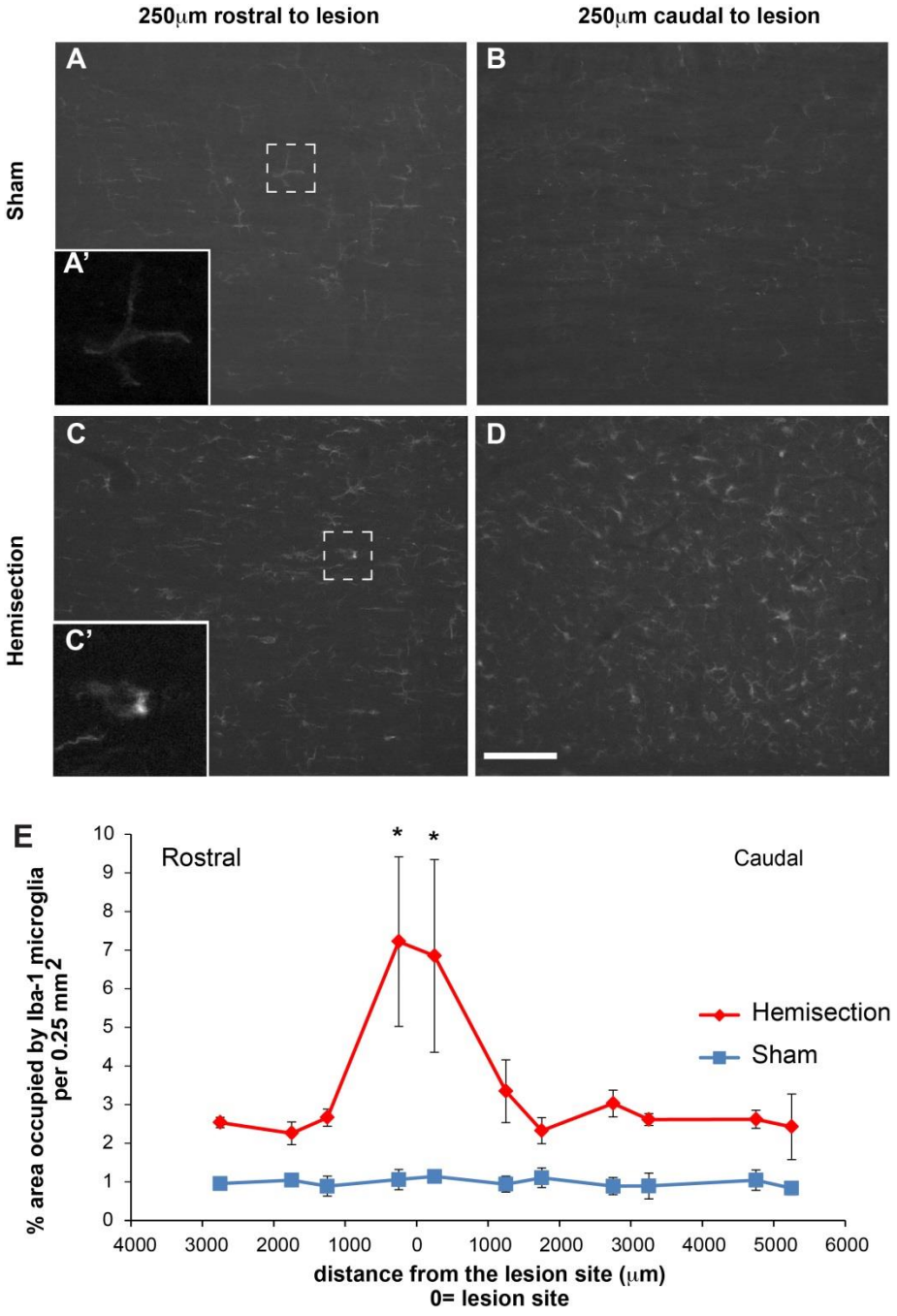

Figure 3-3. Iba-1 staining and quantification of activated microglia in cervical SCI and sham operation animals

Representative images taken from the spinal tissue caudal and rostral to the lesion site show that small resting microglial cells with ramified thin processes were observed in the control group (A,B). Microglia/macrophage activation occurs at 3 weeks after cervical hemisection, with large, rounded Iba-1 labelled cells (C,D). Quantitative analysis showed significant Iba-1 labelling in the epicentre region of cervical hemisection group compared to the sham operation group (* $p<0.05$). Results represent mean ± SEM; n=4 animals in each group. Scale bar =100 μm.

3.3.4 Effect of cervical hemisection SCI on phosphorylated neurofilament

SCI has been reported to result in a significant loss of neurofilaments (Kanellopoulos et al. 2000). In this experiment, the effect of injury on the phosphorylated neurofilament in the cervical spinal cord was examined by using mouse monoclonal antibody (clone SMI-31) in the white matter. In the sham surgery group, SMI-positive axons appeared as numerous, linear and intense staining in the white matter of the spinal cord rostral and caudal to lesion site (Fig 3.4.A,B). In the cervical hemisection group, there appeared to be fewer SMI-31 positive axons in the spinal cord caudal to the lesion compared to the control rats (Fig 3.4.C,D). The analysis revealed a substantial loss of immunoreactivity in the epicenter region (2.8 ± 0.4 vs. 3.9 ± 0.4, $p<0.05$) and caudal part (2.9 ± 0.3 vs. 4.9 ± 1.2, $p<0.05$) of the cervical spinal cord following injury (Fig 3.4E).

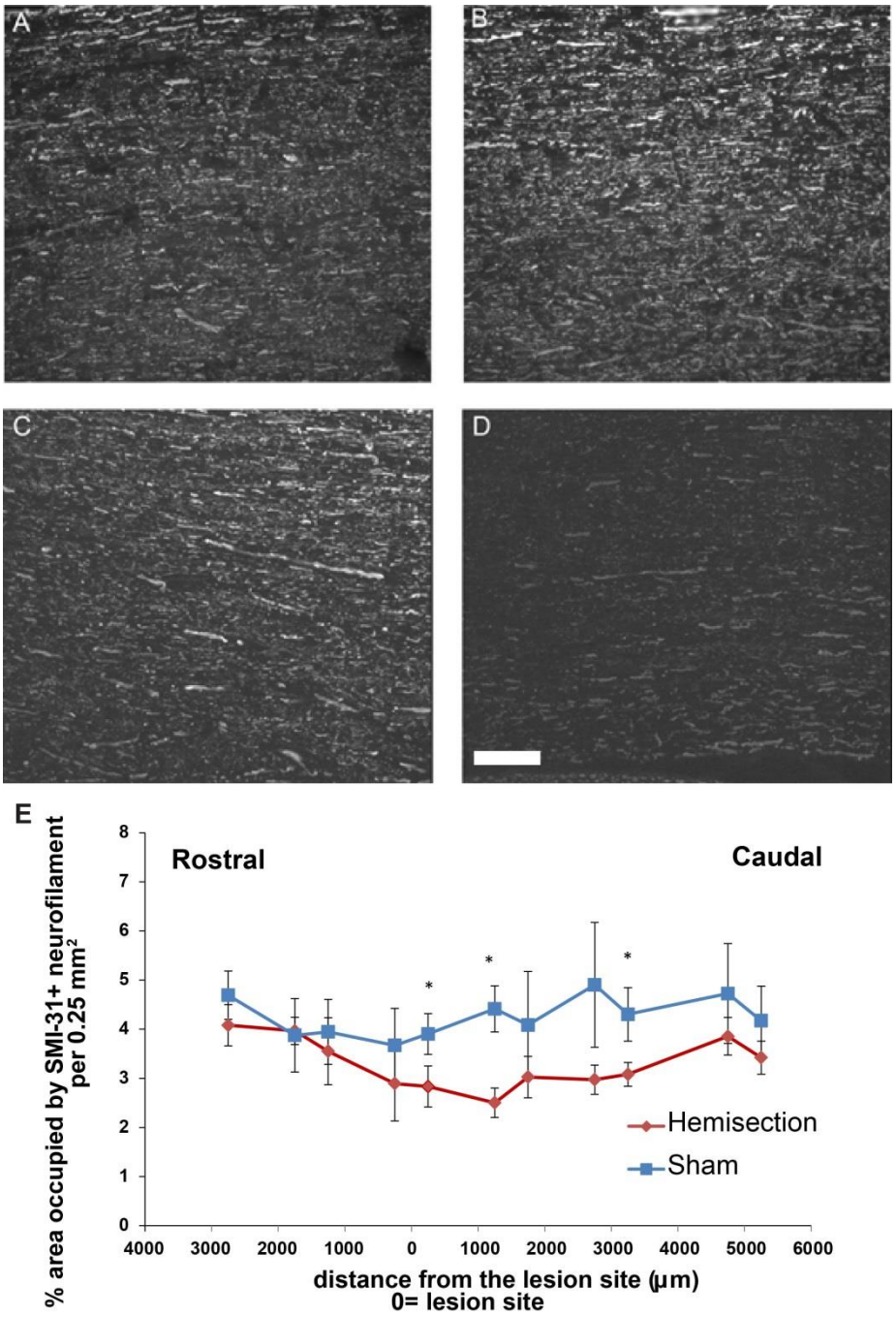

Figure 3-4 SMI-31 staining and quantification of phosphorylated neurofilament in cervical SCI and sham operation animals.
Representative images were taken from the spinal cord rostral (A,C) and caudal (B,D) to the lesion site. In the control group, the SMI-31 labelled axons were arranged in a regular manner. In the hemisection group (C,D), the spinal cord below the lesion site showed less SMI31 immunoreactivity than the control group. There was an overall loss of SMI staining in the cervical spinal cord following cervical hemisection compared to the sham (E) (* $p<0.05$). Result represent mean ± SEM; n=4 animals in each group. Scale bar =100 μm.

3.3.5 Effect of cervical hemisection SCI on serotonin fibres

To assess the depletion of bulbospinal monoaminergic projections after cervical unilateral hemisection, immunohistochemical staining for serotonin was performed at different spinal levels of the animals 1 week and 3 weeks after SCI. The serotonin fibre intensity significantly increased in the vicinity of the lesion site rostral to the lesion compared to the sham group at 1 week and 3 week after SCI (1 week 3.6 ± 0.4, 3 weeks 5.1 ± 1.1 vs. sham 1.4 ± 0.3, $p<0.05$ Fig 3.5B). One week after cervical hemisection, an increase in serotonin fibres rostral, and a decrease caudal to the injury site in the cervical spinal cord was seen. However, the number of serotonin fibres caudal to the lesion site returned to baseline levels by 3 weeks post-injury (Fig 3.5). These data suggest that serotonin fibre sprouting occurs in the absence of any treatment.

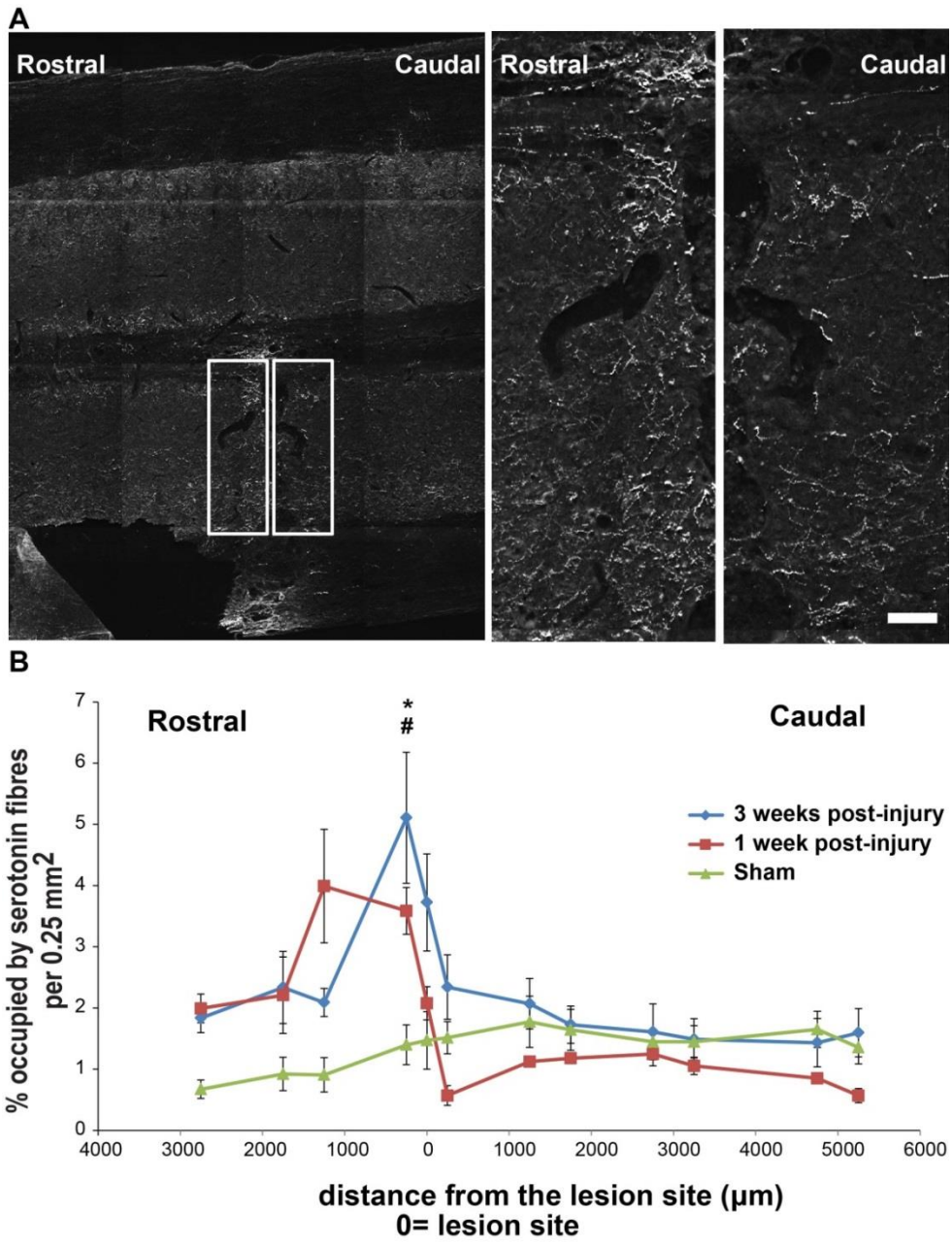

Figure 3-5 Serotonin levels change in the cervical spinal cord rostral to the lesion site

Representative sections (A) showing serotonin axons at the lesion site 3 weeks after cervical hemisection. The boxed areas are shown at higher magnification. Scale bar 100 μm. Quantification (B) revealed serotonin fibres in the rostral part of the lesion site were significantly increased at 1 week (red squares) and 3 weeks (blue circles) compared to the sham operated group after lateral cervical hemisection. Results represent mean ± SEM; n=4 animals per group. However, in the caudal region, 1 week post injury staining appears below the level of the sham operated group. 3 weeks post injury staining is similar to the sham operated group. N = 4 animals per group. # $P<0.05$ 1 week hemisection vs. sham group. * $P<0.05$ 3 weeks hemisection vs. the sham group.

3.3.6 Effect of cervical hemisection SCI on locomotor function

Based on the FLS, the left forelimb function in the hemisection group was severely impaired then improved gradually over a period of 2 weeks following injury (from score 4 to 15). BBB scoring of the hindlimb showed that recovery in the hemisection group developed gradually over 2 weeks following injury (from score 13 to 18, Fig 3.6). The effect of cervical SCI on hindlimb locomotion was not as severe as on forelimb locomotion.

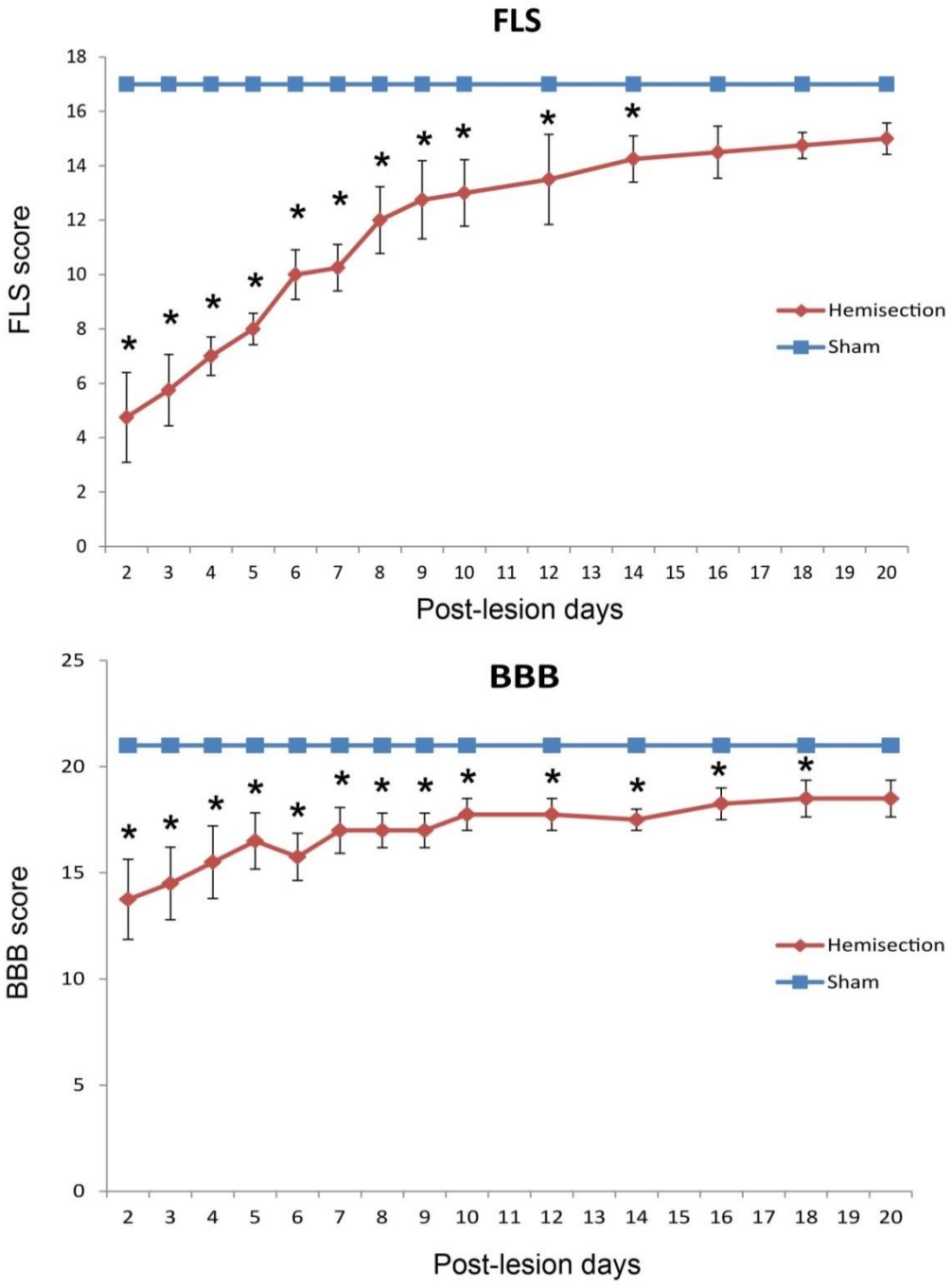

Figure 3-6 Effect of cervical hemisection SCI on locomotor function

Open-field locomotor ability was assessed using the BBB score for hindlimb locomotion. The FLS score was used for forelimb locomotion. A significant difference was observed between the sham and hemisection groups after cervical hemisection (* $p<0.05$). Results represent mean ± SEM; n=4 animals per group.

3.3.7 Effect of cervical hemisection SCI on skilled forelimb function

We used the Montoya staircase test to assess skilled forelimb function. The Montoya staircase test allows assessment of extension and grasping ability independently for each forelimb. For the left forelimb, the staircase study revealed statistically significant differences between the hemisection and sham operation group after surgery (Fig 3.7), for the whole duration of the study.

3.3.8 Effect of cervical hemisection SCI on skilled locomotor function

The grid exploration test was utilized to evaluate the effect of cervical hemisection SCI on skilled locomotor behaviour. The grid exploration test focuses on skilled aspects of limb movements. The animals were placed on the grid for 3 min and allowed to walk freely across the space. After surgery, a significant increase in the misplacement of the paw was observed in the hemisection group following surgery. In contrast, there was no difference in the sham operation group (Fig 3.8).

3.3.9 Effect of cervical hemisection SCI on stepping patterns

To assess stepping patterns of the forelimbs and hindlimbs after injury, animals were required to run along a paper-lined runway to obtain a food treat in a darkened box at the end of the runway. Three different parameters (stride length, stride width, and base of support) were measured. There was a significant decrease in stride length (13.2 ± 1.9 vs.17.5 ±0.5, $p<0.05$) at 2 weeks, with partial recovery by 3 weeks. Left paw stride width decreased gradually after hemisection injury, reaching significance at 3 weeks. The base of support increased at 2 weeks after injury, but recovered by 3 weeks (Fig 3.9).

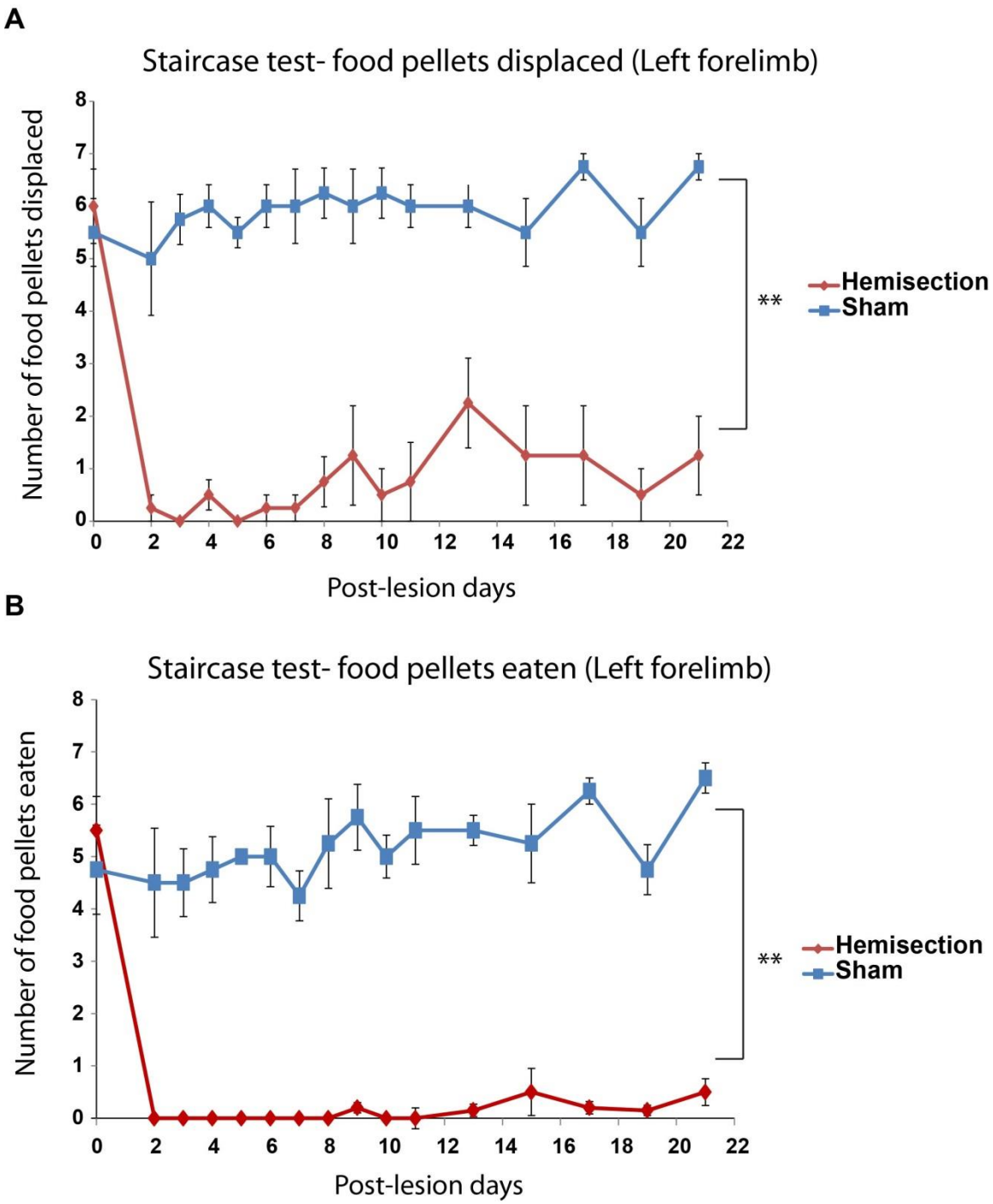

Figure 3-7 Effect of cervical hemisection SCI on skilled forelimb function
Skilled forelimb function was assessed by the Montoya staircase test. (A) The quantification of food pellets displaced on the lesion side revealed a significant loss of the ability to move food pellets after injury and showed little recovery after 3 weeks. (B) The quantification of food pellets eaten using the left forelimb revealed a loss of the ability to retrieve and consume pellets immediately after surgery, and this did not recovery by the end of the experiment.(** $p<0.01$). Results represent mean ± SEM; n=4 animals in each group.

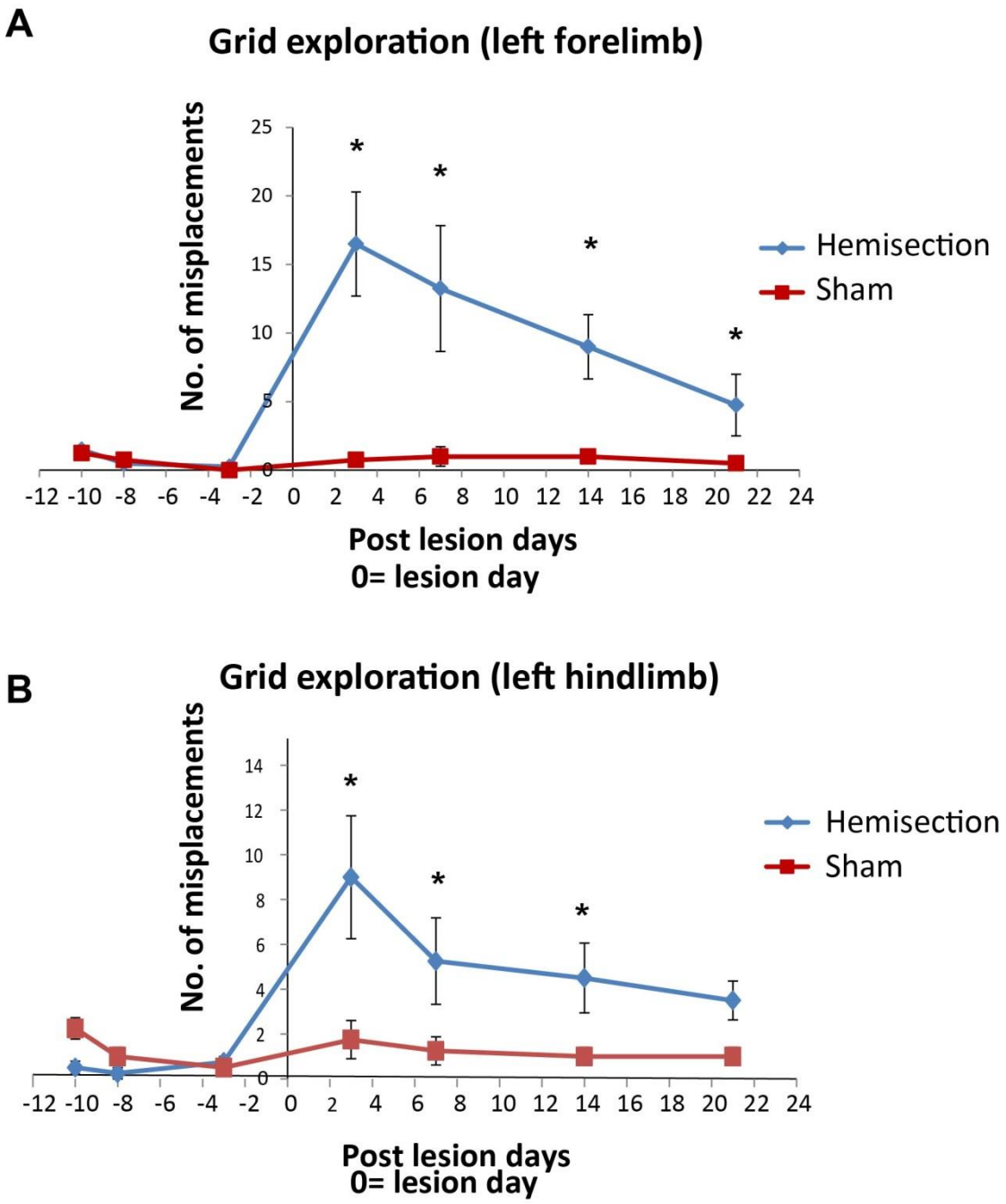

Figure 3-8 Effect of cervical hemisection SCI on skilled forelimb and hindlimb function.

Skilled locomotor function was assessed with the grid exploration test. Three tests were performed before surgery to obtain the baseline data of the grid exploration test. The test was performed at 3 days, 7 days, and 14 days after surgery. After cervical hemisection, the rats made more footslips compared to the sham group in both the left forelimb (A) and hindlimb (B) when walking on the grid. The results revealed highly significant differences between sham and hemisection groups over the study period (* $p<0.05$). Results represent mean ± SEM; n=4 animals in each group.

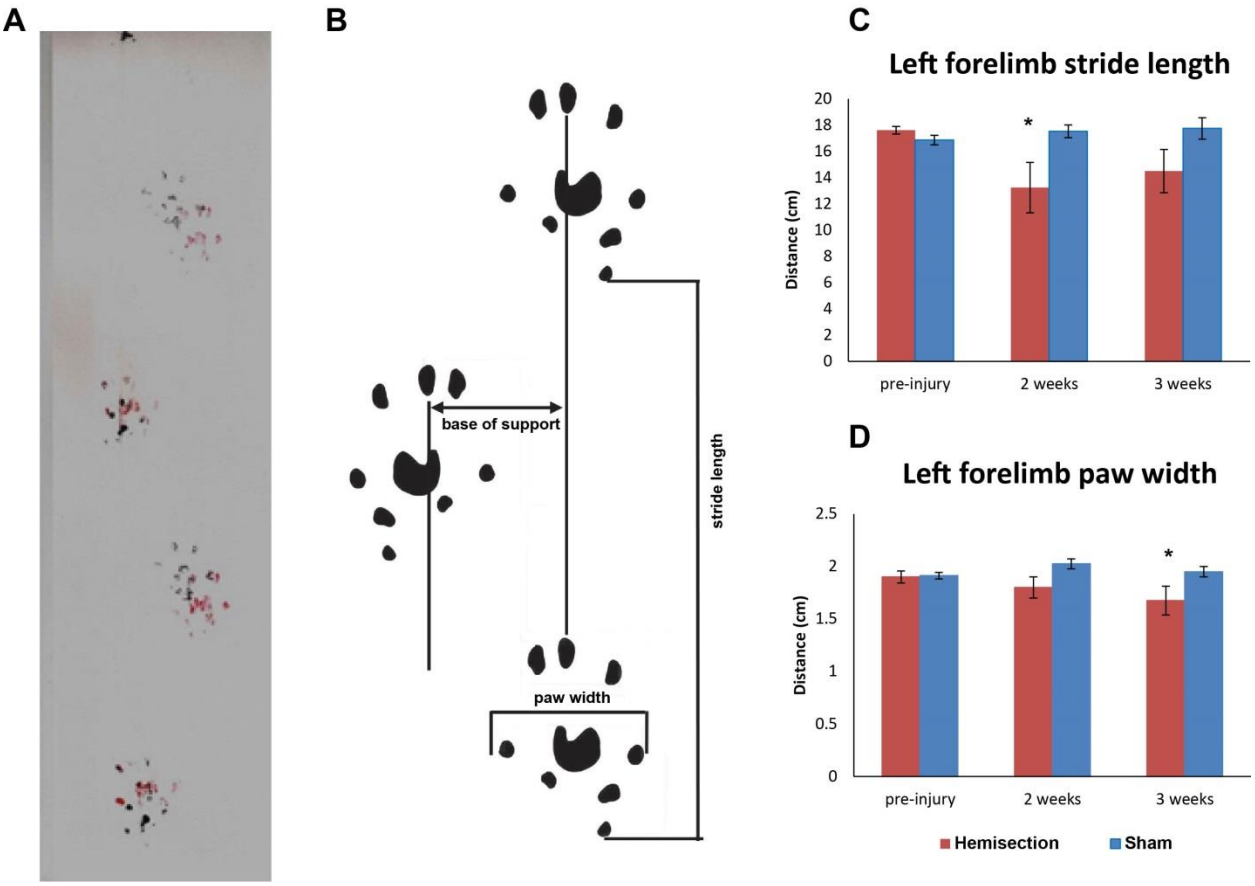

Figure 3-9 Effect of cervical hemisection SCI on stepping patterns

(A) Representative images of the walking track performance in the sham operation group. **(B)** Measurements were taken from the footprints **(C).** The stride length significantly decreased after 2 weeks following SCI compared with the pre-injury stride length. **(D)** Left forelimb paw width decreased after cervical hemisection. The difference reached significance 3 weeks after SCI.

3.4 Discussion

3.4.1 Cervical hemisection SCI animal model

Approximately half of human traumatic SCI occur at cervical segments, causing incomplete lesions in about 70% of cases (Lopez-Dolado et al. 2013). However, most experimental models of SCI have examined thoracic SCI. Histological, behavioural and therapeutic findings in the thoracic SCI model may not be readily applicable to cervical level because of spinal cord diameter differences (Ko et al. 2004), white/gray matter distribution (Wrathall et al. 1995), and the relative dedication of the cord segments to specific ascending and descending systems and specific termination sites (Nathan et al. 1996; Pearse et al. 2005).

Recently, a number of models of cervical SCI have been reported, in which the spinal cord is injured by complete transection, hemisection, contusion, compression, or crush. Different animal models are designed for various study purposes. Complete transection is useful for studying axonal regeneration, while hemisection is optimal for axonal plasticity (Kwon et al. 2002). Furthermore, the hemisection animal model provides a comparison between the injured and non-injury sides in the same animal. For the animal's welfare, given that hemisection results in a less severe injury than complete transection or bilateral injury, postoperative mortality is lower in hemisection compared with complete injury (Kwon et al. 2002; Anderson et al. 2009). Actually, close to two-thirds of all reported human SCI are partial lesions, with the majority occurring in the cervical spinal cord (NSCISC, 2010). In view of clinical translation, the hemisection (partial injury) animal model is closest to the patient injury condition, especially a sharp,

penetrating SCI. In addition, one ultimate aim of our research is to explore plasticity changes after SCI. For this reason the hemisection model is very suitable for our study.

3.4.2 Locomotor function recovery after cervical hemisection

In our study, locomotor function recovered gradually after 2-3 weeks according to the BBB score and the FLS score. The substantial spontaneous recovery of locomotor function over the first month followed by continued improvement has been reported (Webb et al. 2002; Martinez et al. 2009). Recently, a relation between spontaneous locomotor function recovery after SCI and plasticity in spinal neuronal circuits has been generally recognized (Bareyre et al. 2004; Ballermann et al. 2006; Barriere et al. 2008). However, it remains uncertain as to which aspects of plasticity contribute to functional recovery. There are several suggested mechanisms including axonal sprouting (Bareyre et al. 2004; Ballermann et al. 2006), intrinsic re-organization of intraspinal circuits (Barriere et al. 2008) and changes in neurotransmitter systems that facilitate functional recovery, such as glutamate (Giroux et al. 2003), and serotonin (Saruhashi et al. 1996; Schmidt et al. 2000).

3.4.3 Skilled movement recovery after cervical hemisection

Compared to locomotor function recovery, skilled forelimb functions (i.e. staircase test performance) did not recover spontaneously even after 3 weeks. A complete hemisection at C4-5 level interrupts descending motor pathways, including the dorsal CST in the dorsal column and the dorsolateral CST (Casale et al. 1988; Rouiller et al. 1991), which are important for distal flexor function. In addition to gripping ability, food

pellet retrieval requires postural adjustments, forearm extension, grasping with the digits, and supination and flexion to bring the food to the mouth. This kind of function requires fine, skilled movement of the fingers and the behaviour is quite similar to humans (Whishaw et al. 1992). Complete hemisection at C5 cervical level causes a loss of gripping ability in the ipsilateral forepaw in rats, and the impairment is permanent (Anderson et al. 2005; Anderson et al. 2007). Another interpretation is that the hemisection lesion creates a disruption to the rubrospinal tract, which results in impaired movement of the distal forelimbs and significant deficit in digit flexion (Schrimsher et al. 1993). In addition to the lesion of descending fibres resulting in impairment of food retrieval, one study found that the sensory input conveyed by the dorsal columns is important for both proximal and distal limb movements used for skilled reaching (McKenna et al. 1999). Further work has demonstrated that rats with unilateral cervical dorsal column lack the ability to discriminate surfaces of different textures (Ballermann et al. 2001). Skilled forelimb behaviours require the integration of both sensory and motor systems. Because of this complexity, skilled movement can be used as a sensitive indicator of the therapeutic effects of novel therapies.

3.4.4 Histology findings after cervical hemisection SCI

In my animal model, it is likely that the cervical hemisection SCI leads to neuronal, oligodendrocyte, and phosphorylated neurofilament loss at the lesion site. Most neuronal loss is considered to be the result of necrosis caused by the original injury (Wrathall 1992). However, a number of other studies have shown the occurrence of neuronal apoptosis after SCI (Bareyre et al. 2003). Secondary injury following the

primary insult such as inflammation, ischaemia, glutamate-induced toxicity, and free radical production, could contribute to neuronal loss. Some studies suggested that loss of NeuN immunoreactivity after CNS injury does not indicate neuronal cell loss but rather suppression of the antigen in intact neurons (McPhail et al. 2004; Unal-Cevik et al. 2004). However, one study demonstrated the loss of neurons in ventral horn after SCI with toluidine blue, which is compatible with the pattern of NeuN expression, suggesting that NeuN loss does represent actual neuronal loss following SCI (Huang et al. 2007).

Oligodendrocytes are the cells responsible for the myelination of axons in CNS, and remyelination of injured axons. APC is a marker that has been identified as a useful oligodendrocyte marker. APC clearly labels the oligodendrocyte cell body, but not processes or myelin sheaths (Bhat et al. 1996). In comparison with myelin protein antibodies, the ability of APC to label the oligodendrocyte cell bodies without labelling processes or myelin offers unique advantages for the study of oligodendrocytes. We found loss in the number of oligodendrocytes after cervical SCI compared to the sham operation group. These findings are consistent with other SCI studies (Liu et al. 1997).

To investigate axonal pathology, a neurofilament maker (SMI 31) was examined in my animal model. Our data revealed a decrease in the phosphorylated component of neurofilament 3 weeks following SCI, especially in the epicenter region and caudal to the lesion site. This finding is consistent with reports on a decrease in phosphorylated and non-phosphorylated neurofilament protein following SCI (Kanellopoulos et al. 2000; Ward et al. 2010). The primary function of neurofilament is to maintain the axonal caliber. Destruction of this protein will lead to a dramatic decrease in the caliber of

spinal cord axons and reduce their conduction velocity (Liu et al. 2004).

3.4.5 Serotonin changes associated with SCI

Serotonin may have a biphasic influence on spinal cord recovery after trauma. A microdialysis study has demonstrated that large amounts of serotonin are released acutely in the injured spinal cord (Sorkin et al. 1991). Serotonin is released from the neural tissue at the injury side and is transiently taken up by platelets (Saruhashi et al. 1991). It has been proposed that the release of serotonin in the acute stage of SCI contributes to local adverse effects such as the traumatic decline in blood flow and the oedema in injured cords (Sharma et al. 1990).

It has been reported that rapidly diminished spinal levels of serotonin occur ipsilateral to spinal lesion following thoracic SCI, with levels of serotonin returning by 4 weeks after injury in the lumbar region (Saruhashi et al. 1996; Hains et al. 2002; Saruhashi et al. 2009). These dynamic changes can be used as an indicator of injury severity (Faden et al. 1988) and provide a substrate for changes in somatosensory and locomotor behaviours. There are two considerations which could contribute to the recovery of serotonin levels after SCI. Although most serotonin pathways descend ipsilaterally, it would appear in some immunohistochemical studies that serotonin fibres cross in the region of the central canal (Hadjiconstantinou et al. 1984; Saruhashi et al. 1996). Secondly, Newton and Hamill report some serotonin neurons found in lamina VII and X of the thoracolumbar and sacral spinal cord of the adult rat (Newton et al. 1988). Such intraspinal serotonin neurons may be a source of fibres that reinnervate the spinal cord.

In cervical SCI animal models, there is limited data about the changes that occur in serotonin levels at the lesion site and how they correlate to behavioural recovery. In my

animal model, the serotonin level was markedly increased in the cervical spinal cord rostral to the lesion site even 3 weeks after injury. It is postulated that serotonin contributes to neuroplasticity at the lesion site and accumulates in the descending bulbospinal serotonergic pathways that are cut by the cervical hemisection. In contrast, the serotonin level in the spinal cord caudal to lesion site showed no obvious difference from the control group.

3.5 Summary

- A cervical spinal cord hemisection injury model has been successfully developed in the rat, which revealed a strong correlation between histological finding and neurological recovery.
- The behavioural tests showed substantial spontaneous recovery of crude forelimb function, but a long-lasting deficit in fine forelimb function.
- Neuronal and oligodendrocyte loss after cervical hemisection injury was observed, which offers an opportunity for neuroprotective intervention.
- Microglia/macrophage activation was observed after injury. These findings are consistent with other SCI animal models.
- A substantial loss of phosphorylated neurofilament was observed 3 weeks after SCI.
- Hemisection led to neuroplastic changes in the cervical spinal cord, including increased expression of serotonin fibres at the lesion site.
- In conclusion, our animal model is a reliable animal model to assess the pathophysiological mechanisms of cervical SCI and can be used to investigate

neuroprotection and neuroplasticity in subsequent work.

4 Neuroprotective effect of DHA treatment in cervical hemisection SCI

4.1 Introduction

4.1.1 Neurological benefits of DHA

The first study with DHA treatment in traumatic CNS injury used a model of thoracic hemisection SCI in adult rats (King et al. 2006). Evidence of neuroprotective potential has been provided involving a variety of mechanisms, including anti-inflammatory effects through inhibition of the production of proinflammatory cytokines (Endres et al. 1996), antioxidant effects (Sarsilmaz et al. 2003), and apoptosis prevention (Martin et al. 2002). Both neurons and oligodendrocytes are highly vulnerable to cell death following SCI (Bunge et al. 1993). Work in our laboratory has shown that omega-3 PUFAs have potent neuroprotective effects. In the first of our studies, an intravenous bolus of DHA was given 250 nmol/kg 30 min after thoracic hemisection injury (King et al. 2006). The results showed that DHA can decrease the neuronal cell and oligodendrocyte loss, and reduce apoptosis following injury. Then, we studied a more severe SCI type, compression injury, in rat thoracic spinal cord (Huang et al. 2007). Similar to what was seen in hemisection injury, the DHA treatment reduced neuronal cell loss and oligodendrocyte loss. Furthermore, the administration of DHA after injury also reduced the activation of microglia and macrophages, which suggested that DHA can suppress the inflammatory response. Concerning the axonal injury, we found that DHA can decrease the number of injured axons labelled with β-APP, which is anterogradely transported under normal conditions, but abnormally accumulates in axons following

injury (Huang et al. 2007). These histological findings were supported by the improved recovery of locomotor function after DHA treatment. In addition, the neuroprotective effect of acute DHA treatment (500 nmol/kg) was also demonstrated in another species, the mouse, following compression injury (Lim et al. 2013). Following mouse compression injury, we found that DHA also reduced neuronal, oligodendrocyte and neurofilament loss and suppressed macrophage/microglial activation in both the grey and white matter. The promising neuroprotective effects of DHA were also demonstrated in other laboratories. Pretreatment with DHA of rats subjected to thoracic contusion SCI increases the preservation of axons and survival of neuronal cells and oligodendrocytes (Figueroa et al. 2012). Dietary omega-3 PUFA prophylaxis also accelerates bladder recovery, improves locomotor function and ameliorates sensory dysfunction (Figueroa et al. 2013).

4.1.2 DHA treatment in cervical SCI

The neuroprotection effect of DHA was well demonstrated in thoracic SCI rodent animal models. However, the majority of reported human injuries occur at cervical level. There are several anatomical differences between the thoracic spinal cord and cervical spinal cord, such as differences in spinal cord diameter, white/grey matter distribution, the degree of vascularization, and the targets of innervation (Pearse et al. 2005). The therapeutic effect of DHA in thoracic SCI may not be applicable to cervical SCI. Before DHA is tested in clinical trials, it should be validated in a wide range of preclinical SCI models, including a cervical SCI model.

Furthermore, rats do not use their hindlimbs as skillfully as their forelimbs, nor can use

of their hindlimb paw and digit be as carefully evaluated as the forelimb paws and digits. Compared to thoracic SCI, recovery from forelimb dysfunction offers more general and skilled motor movements by which to better assess the efficacy of potential therapies, especially mild degrees of improvement (Soblosky et al. 2001).

4.2 Aims

Confirmation of efficacy in more than one species or model is one of the criteria for consolidation of the translational value of a treatment in SCI. Our hypothesis in this chapter is that DHA can improve neurological function recovery and histological preservation in cervical hemisection animal model. Therefore, the present study was designed to further investigate the effects of DHA using a cervical hemisection SCI model. The therapeutic effect of DHA was assessed by histological analysis and behavioural tests.

4.3 Results

4.3.1 DHA treatment increased neuronal cell survival after spinal cord hemisection injury

Examination of NeuN labelling at 21 days post-injury revealed that rats that received DHA treatment after injury had substantially more labelled cells both rostral and caudal to the lesion site (Figure 4.1). The quantitative analysis of NeuN positive cells confirmed the significant differences; rats in the DHA treatment group had more NeuN labelled cells in the region within 1250 μm caudal and rostral to the lesion site (71.3 ± 7.4 vs.44.3 ± 6.7 caudal to the lesion site, 58.2 ± 5.8 vs. 39.7 ± 11 1250 μm rostral to the lesion site, $p<0.05$ Fig 4.1). There was no statistically significant difference in NeuN-labelled cells in the more caudal part of the spinal cord between the two groups. The results suggest that DHA has a neuroprotective effect in the vicinity of the lesion site.

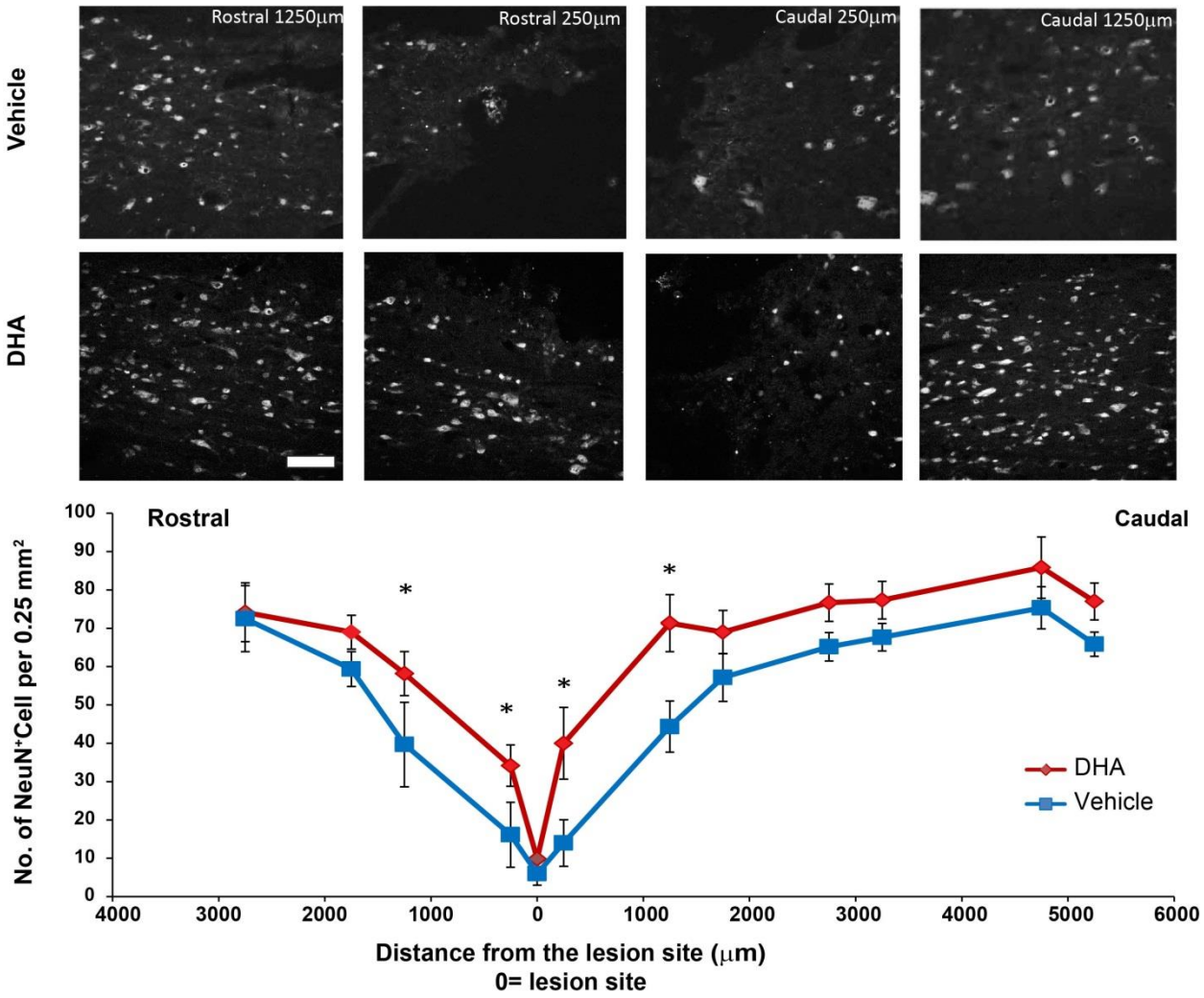

Figure 4-1 Effect of the acute administration of DHA on NeuN staining after rat cervical hemisection

Images show representative neuronal (NeuN) labelling in the cervical spinal cord in the vehicle and DHA treatment groups. Scale bar =100 µm. Quantification revealed there was a significant difference in the epicentre region (*P<0.05). Results represent mean ± SEM; n=6 animals in each group.

4.3.2 DHA treatment has a modest effect on oligodendrocyte survival after spinal cord hemisection injury

In the epicentre of spinal cord lesion, APC-labelled oligodendrocytes were lost in both groups. From the images captured from the vicinity of the spinal cord lesion, there appeared to be slightly more APC-labelled oligodendrocytes in the DHA treated group (Fig. 4.2). Regarding the quantification of APC-labelled oligodendrocytes in the perilesional area, there was no significant difference between DHA and the vehicle group (Fig 4.2).

4.3.3 DHA treatment ameliorates neurofilament loss

SMI31 was used to detect phosphorylated neurofilament. At 3 weeks after cervical hemisection, rats that received acute DHA administration 30 min after injury had substantially more SMI-31 labelled axons in the spinal cord caudal to the lesion site (Fig 4.3). The quantification of SMI31 positive neurofilament revealed no difference in the vicinity of the lesion site (5.9 ± 1.1 vs. 5.1 ± 0.8 in the lesion site). However, in the spinal cord 3000 μm below the lesion site, the immunoreactivity of SMI31 in white matter in the DHA group was 2 times more than in the vehicle group (9.3 ± 2.0 vs. 4.9 ± 0.8 $p < 0.05$).

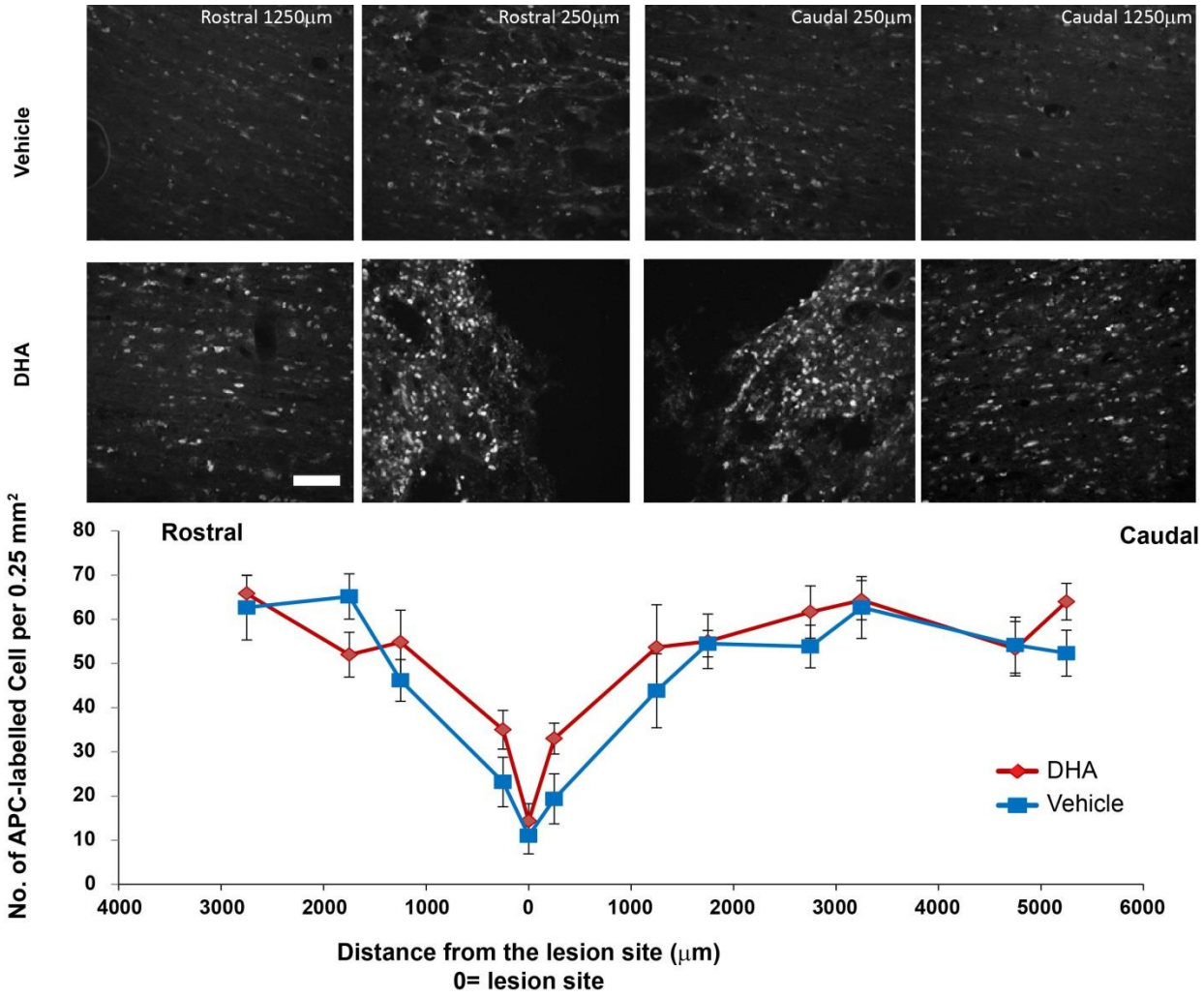

Figure 4-2 Effect of the acute administration of DHA on APC staining after rat cervical hemisection.

Images show representative APC labelled oligodendrocytes in the white matter of the vehicle and DHA groups. Scale bar = 100 μm. Quantification of APC labelled cells revealed more oligodendrocyte survival in the epicentre region of the DHA group. However, the difference was not significant ($p>0.05$). Results represent mean ±SEM; n=6 animals in each group.

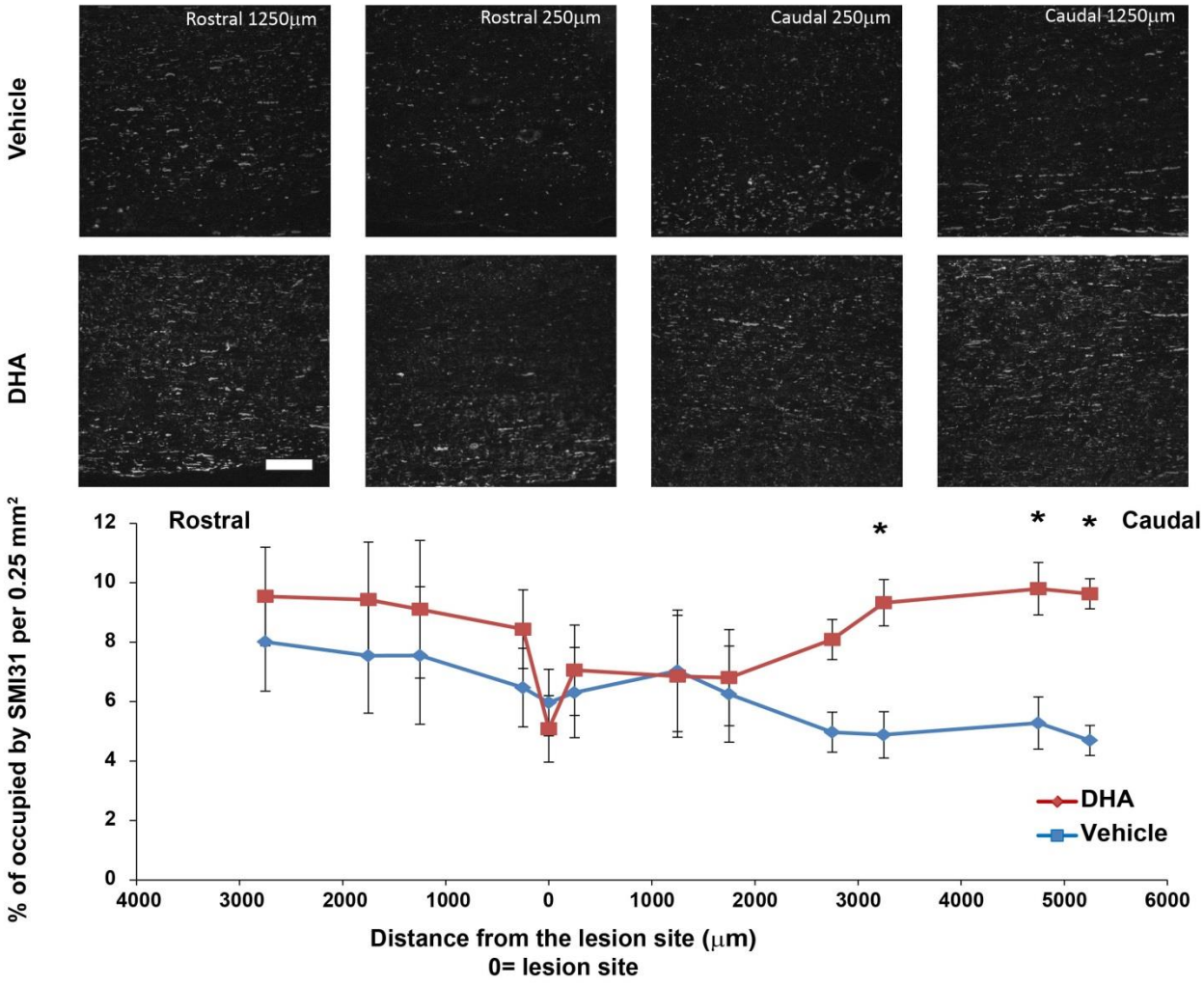

Figure 4-3 Effect of the acute administration of DHA on SMI-31 labelling of axons after rat cervical hemisection.

Representative images showed SMI-31 labelled axons at various levels caudal and rostral to the lesion site in vehicle and DHA-treated groups. Scale bar=100 μm Quantification revealed significant differences in the amount of SMI-31 labelled axons below the level of 3,000 μm caudal to the lesion site between the two groups. Results show the mean ± SEM of n=6 animals in each group.

4.3.4 DHA treatment decreases microglial staining

Iba-1 is an antibody commonly used to identify resting and activated microglia in rat spinal cord tissue. The micrographs revealed a substantial increase in the size and number of Iba-1 cells in both groups (Fig 4.4). Overall, the microglial cell staining was higher in the vehicle group than the DHA treatment group, with a significant difference at the epicentre (7.6 ± 0.9 vs. 5.7 ± 1.3, $p < 0.05$).

4.3.5 DHA treatment decreases the lesion size

GFAP labelling at the spinal cord hemisection site in both groups revealed the lesion epicentre as an area devoid of GFAP staining (Fig 4.5). The lesion extended from the midline to the lateral edge of the spinal cord. Quantitative analysis showed that the DHA treatment reduced the lesion size by 50% compared to the vehicle group, but the difference did not reach statistical significance.

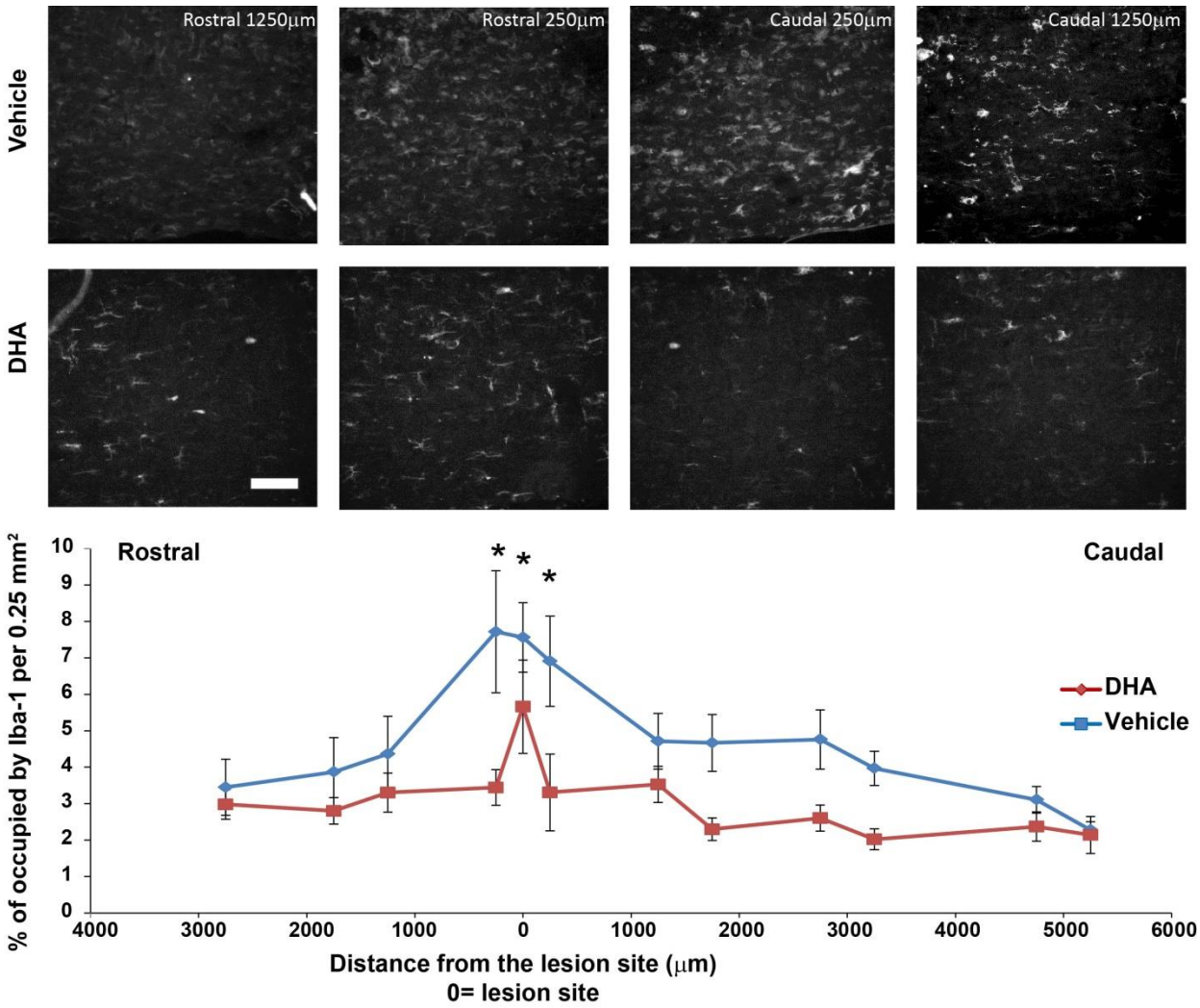

Figure 4-4 Effect of the acute administration of DHA on activated microglia after rat cervical hemisection SCI.

Images show Iba-1 immunoreactive cells at various levels rostral and caudal to the lesion site in vehicle and DHA-treated groups. Scale bar=100 µm. Quantification revealed there was a significant difference in the epicentre region (*P<0.05). In the DHA treatment group, the Iba-1 immunostaining was lower than in the vehicle group. Results represent mean ±SEM; n=6 animals in each group.

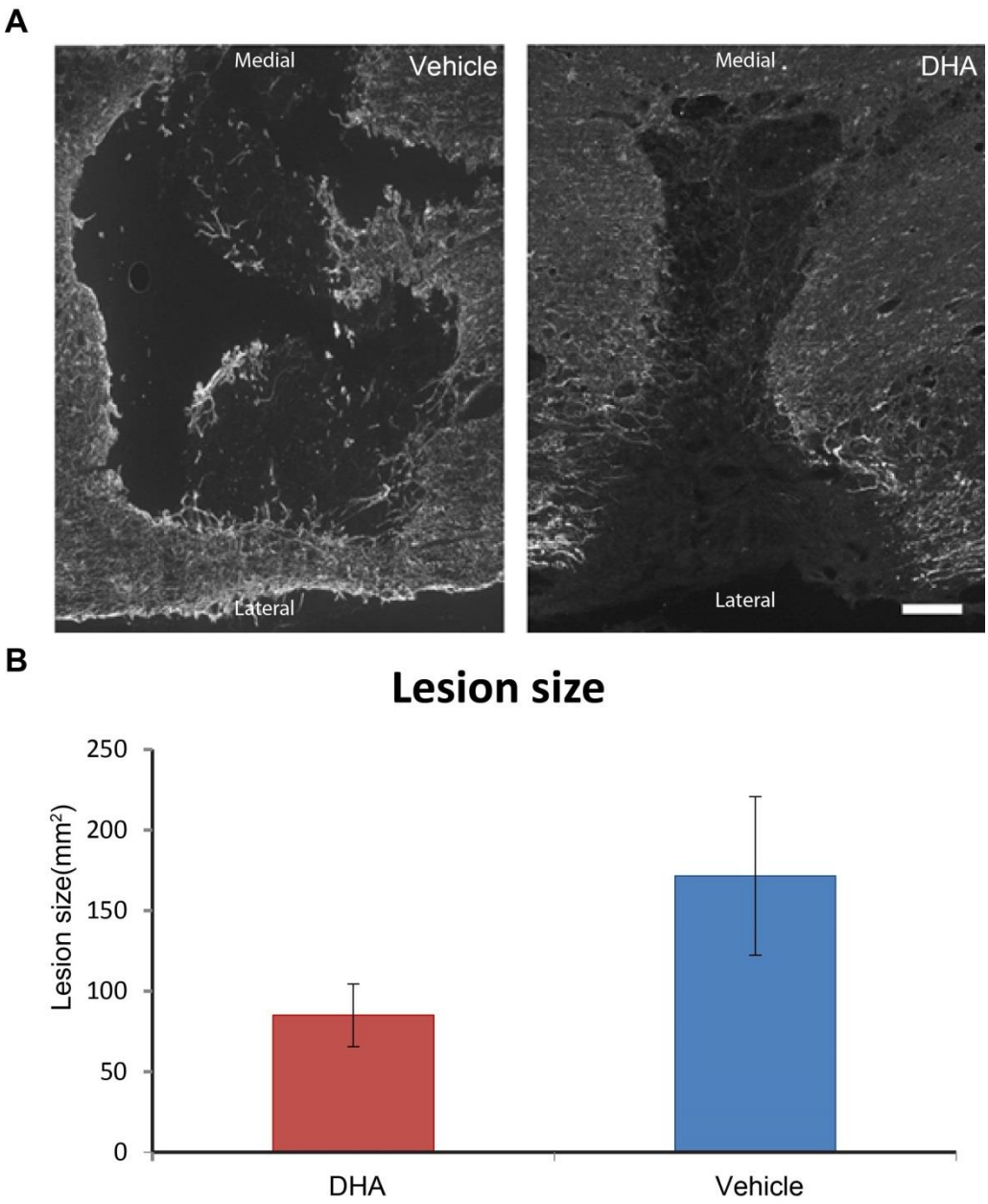

Figure 4-5 Effect of DHA treatment on lesion size

(A) GFAP stained spinal cord sections from vehicle and DHA treated rats show a difference in lesion size and morphology. In DHA treated rats, the lesion margin was well defined compared to vehicle group. Scale bar = 100 μm (**B)** Quantification revealed DHA decreased the lesion size, but there was no significant difference ($p=0.133 > 0.05$) vs. vehicle treated rats. Results represent mean ±SEM; n=6 animals in each group.

4.3.6 DHA treatment improves locomotor behaviour recovery

To determine the general ability of the animals to use their forelimbs and hindlimbs, all animals were tested in the open field test 1 day after cervical spinal cord hemisection. The animals treated with DHA had the best locomotor outcome overall (Fig 4.6). The ANOVA analysis revealed that there was a significant treatment effect within the first week after cervical hemisection, especially significant on days 1, 2, and 3 on BBB scores, and days 1 and 2 on FLS scores. In general, all animals recovered gross hindlimb and forelimb motor function within 2 weeks with and without treatment, which is comparable with other studies (Wang et al. 2011; Khaing et al. 2012). However, the quantitative data revealed a trend showing that rats treated with DHA had higher scores in FLS and BBB than the vehicle control group, and maintained their improvement until the end of the study.

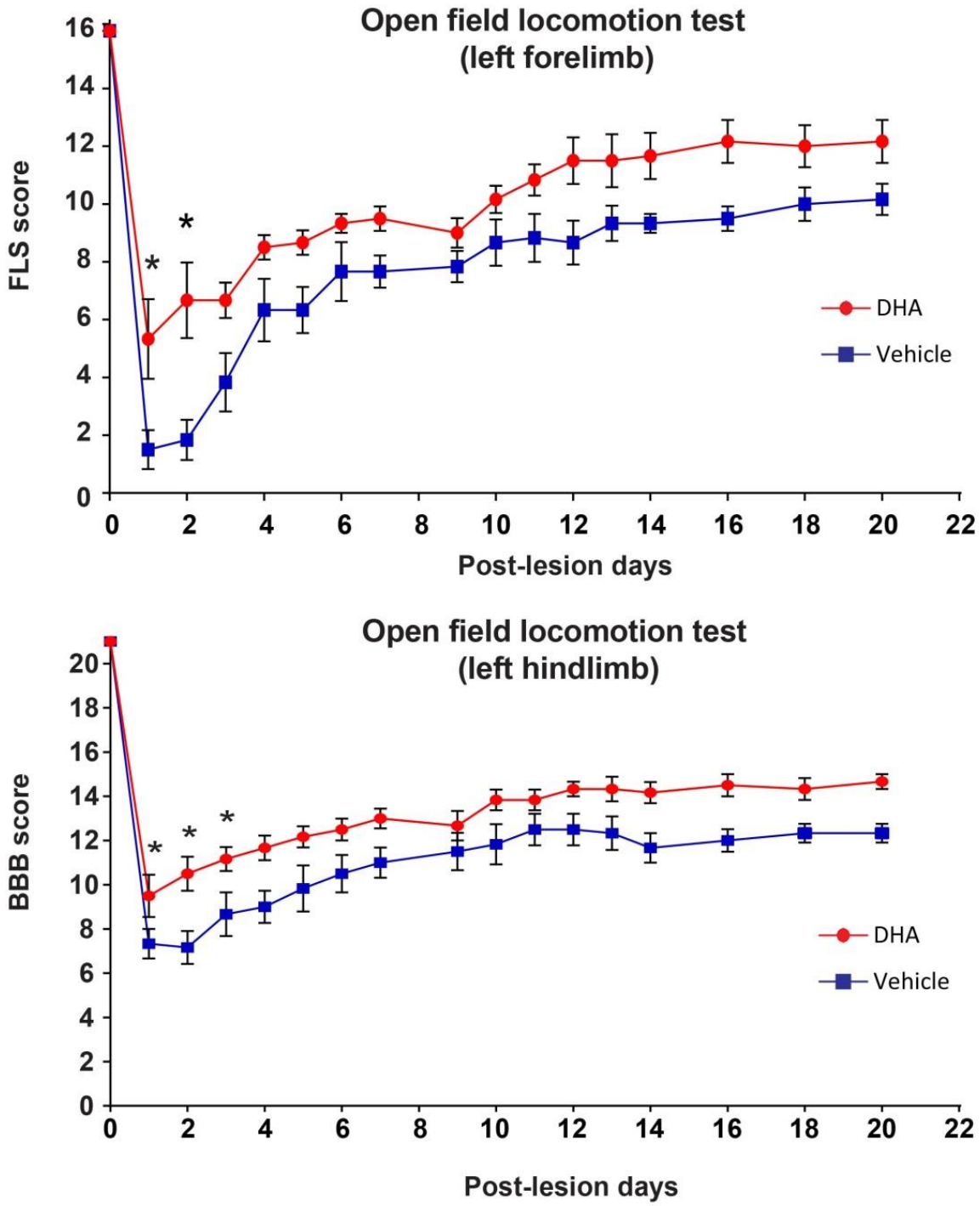

Figure 4-6 The effect of treatment with DHA on locomotor recovery after cervical hemisection SCI

Statistical analysis showed that DHA treated animals had significantly higher scores on ratings of hindlimb locomotor use in the open field BBB test than the vehicle group during the first, second, and third day after surgery. The FLS revealed a significant difference during the first and second day after surgery. Results represent mean ±SEM; n=6 animals in each group.

4.3.7 DHA treatment improved skilled forelimb function

To assess skilled forelimb motor function, the dexterity of the affected forepaw was examined using the staircase test. All animals received training on the staircase task before injury, and only animals that performed successfully on the staircase (successful retrieval of 6 out of 7 food pellets) were included in the study. We used two parameters to assess the forepaw function: the number of food pellets eaten and the number of pellets that were displaced but not eaten by rats.

Concerning the number of pellets retrieved, one day after SCI injury, all rats demonstrated a marked drop in grasping performance and in the number of food pellets retrieved. After one week post injury, food pellets were displaced but not eaten in the DHA treatment group (Fig. 4.7). Two weeks later, significantly more food pellets were eaten by the DHA treated rats compared to the vehicle control group (0.88 ± 0.6 vs. 0 ± 0, $p<0.05$ Fig. 4.7). Grasping performance gradually improved in the DHA-treatment group, and this was significant after 20 days post-injury. On the last testing day, the animals treated with DHA seemed to still follow a trajectory of improvement.

4.3.8 DHA treatment improved skilled locomotion

During the 3 week period after cervical hemisection, the grid exploration test was used to assess skilled locomotor function. In the grid exploration test, all the animals gradually recovered forelimb placement 1 week, 2 weeks, and 3 weeks following SCI. A significant difference was observed in limb misplacement between the DHA treated group and the vehicle group (Fig. 4.8). The animals with DHA treatment exhibited significantly fewer misplacements than the vehicle group in both forelimb and hindlimb at 1, 2 and 3 weeks post-injury.

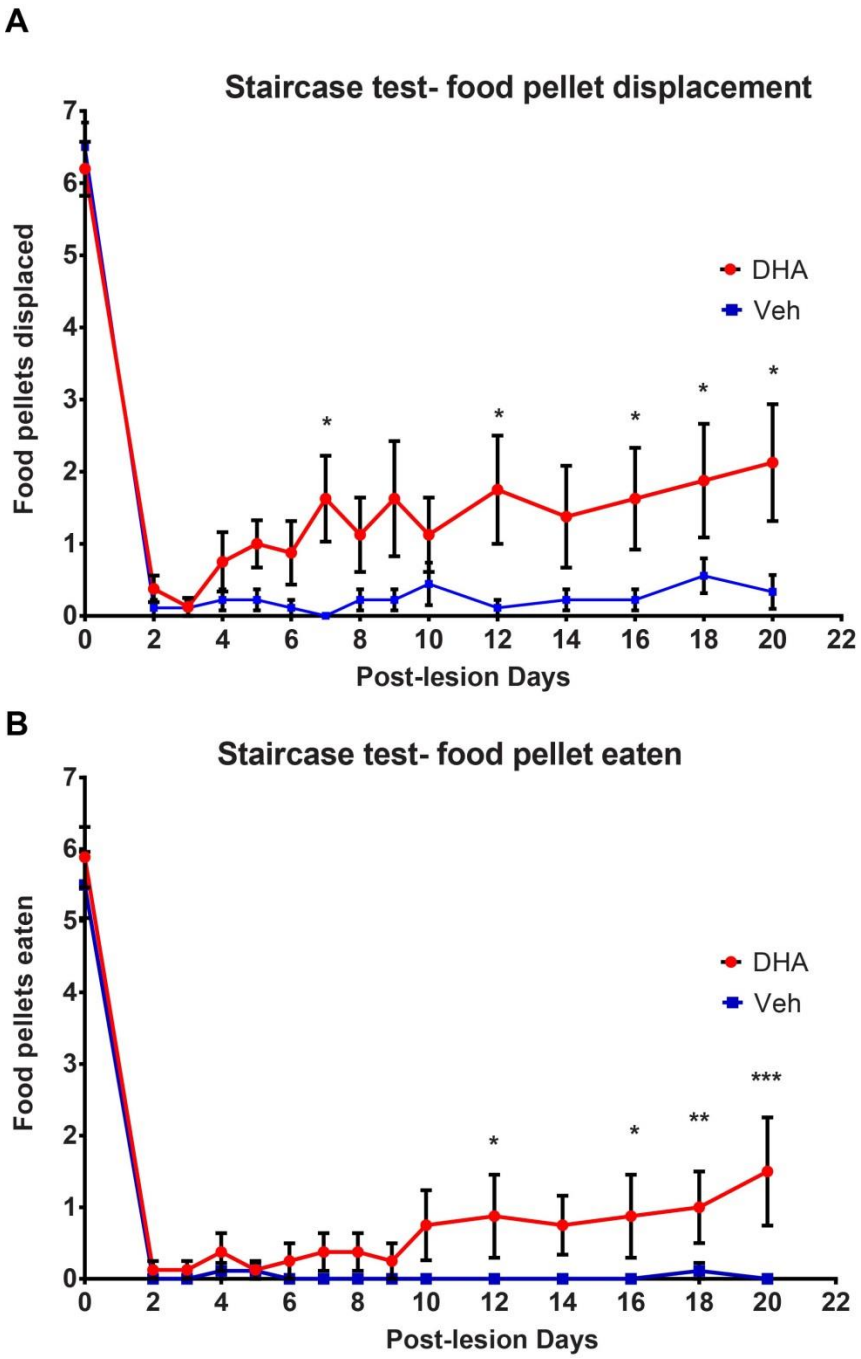

Figure 4-7 Effect of DHA treatment on skilled forelimb function.
Skilled forelimb function was assessed by the Montoya staircase test. (A) All animals lost the ability to retrieve the food pellet after cervical hemisection. The animals treated with DHA gradually recover food retrieval ability. A significant difference was observed between the groups. (B) Displacement indicates that the rat had the ability to reach the food pellet, but failed to put the food pellet into their mouth. Quantitative analysis shows that the animals in DHA treatment group have more ability to displace the food pellet. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ DHA vs. vehicle group. Results represent mean ±SEM; n=8 animals in each group.

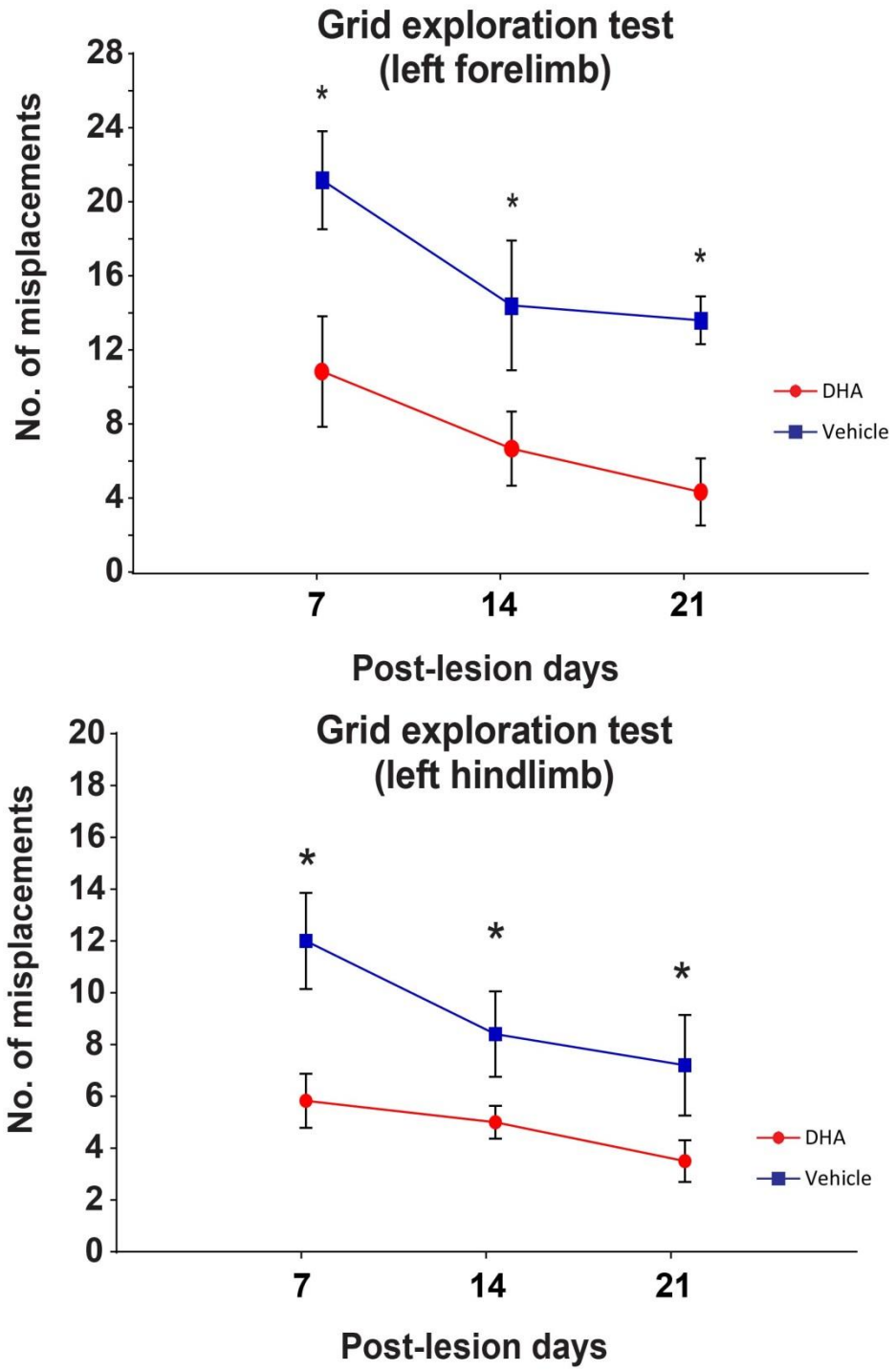

Figure 4-8 Effect of cervical hemisection SCI on skilled locomotor movement
Skilled locomotion was evaluated by grid exploration test 1, 2 and 3 weeks after cervical hemisection. After injury, the animals treated with DHA made significantly fewer forelimb and hindlimb mistakes compared to animals receiving saline injection. Quantification of left forelimb and hindlimb misplacement shows the number of misplacements is significantly higher in the vehicle group than in the DHA treatment group (* $P<0.05$). Results represent mean ±SEM; n=6 animals in each group.

4.3.9 DHA treatment dose not promote functional recovery when administrated in the subacute phase of SCI

To investigate the effect of DHA on skilled forelimb function in the subacute stage following SCI, DHA was administered via tail vein injection 3 weeks following cervical spinal cord hemisection. Skilled forelimb and hindlimb function was assessed by the Montoya staircase test and the grid exploration test. In the Montoya staircase test, all rats lost the ability to retrieve the food pellets after cervical SCI. Sixty-two days after SCI, the DHA-treated group still cannot regain the ability to eat or displace more food pellets than the vehicle group (Fig 4.9). In the grid exploration test, the results showed no significant difference in misplacements made (left forelimb and hindlimb) between the two groups, before and after DHA injection (Fig 4.10). Therefore, there is no therapeutic effect of the DHA treatment delivered at the subacute stage in cervical SCI.

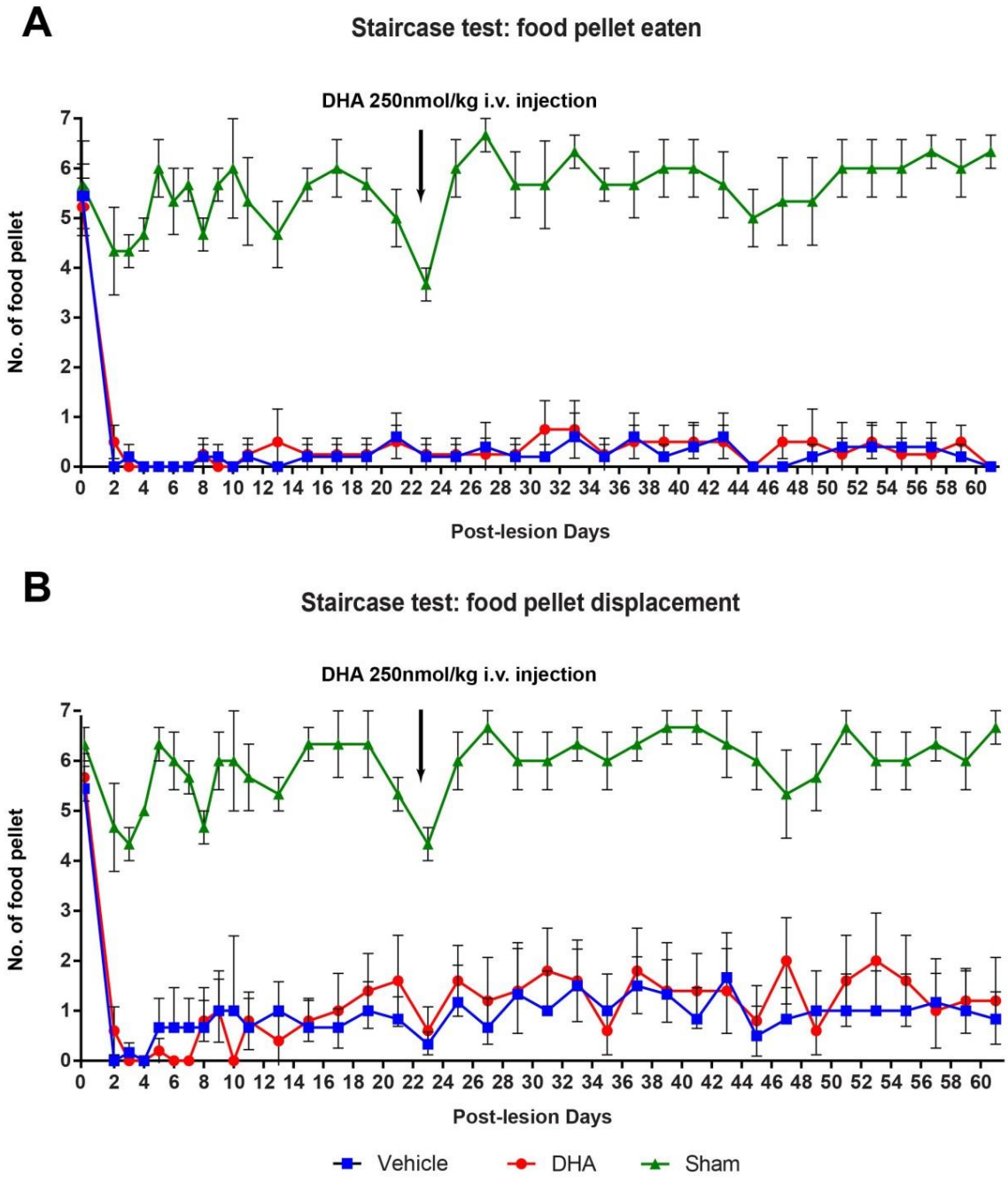

Figure 4-9 Delayed DHA treatment does not promote improved forelimb skilled functional recovery

(A,B) In the Montoya staircase test, all injured animals lost the ability to displace (gross motor function) and eat (fine motor function) the food pellet after cervical lateral hemisection, from 2 days post injury. The animals treated with either vehicle (blue square) or DHA (red circle) at 3 weeks post injury did not significantly recover food retrieval ability in comparison to uninjured sham operated animals. Results represent mean ±SEM; n=5-6 animals in each group.

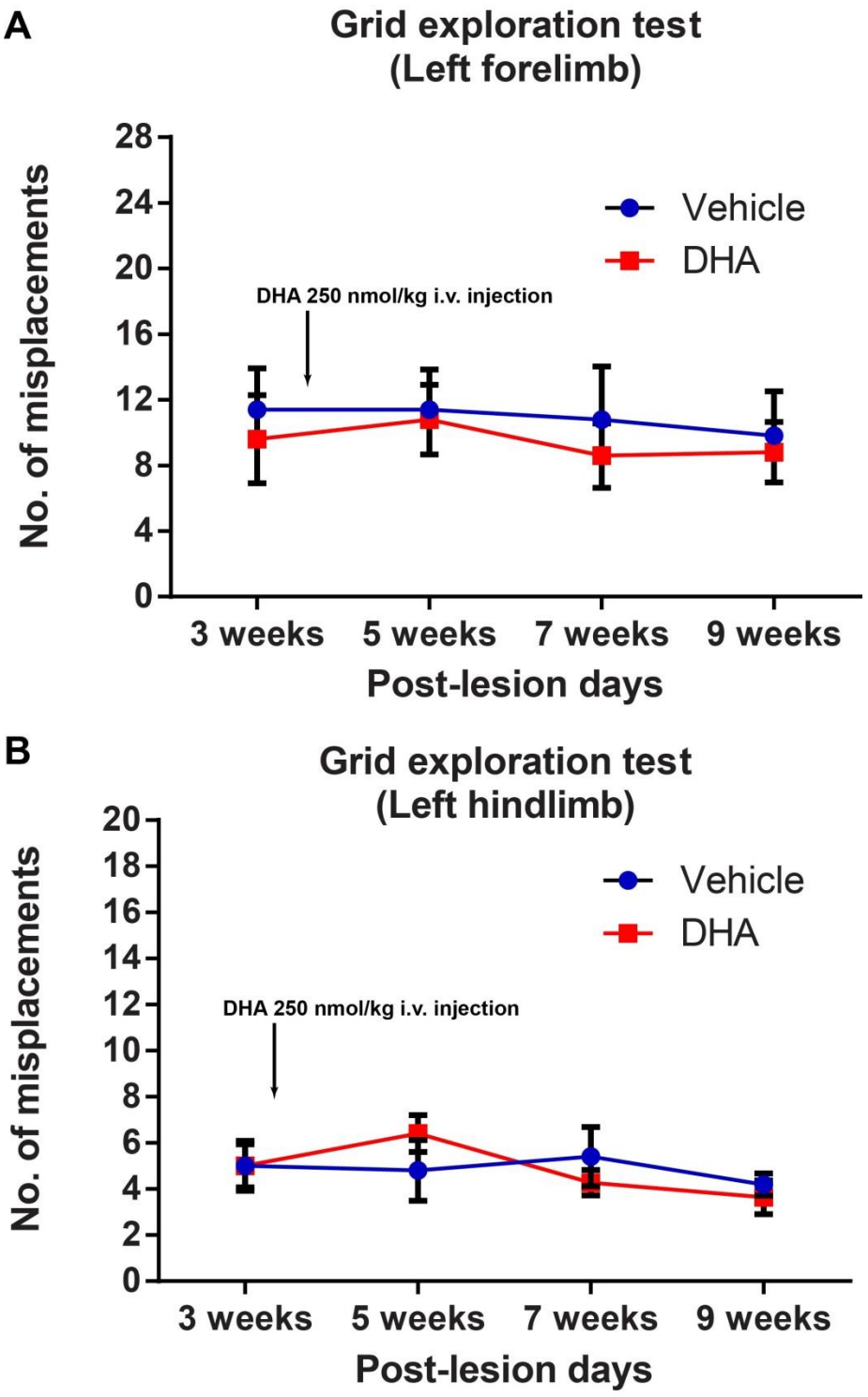

Figure 4-10 Delayed DHA treatment does not improve skilled locomotor recovery

DHA treatment was given 3 weeks after cervical spinal hemisection. In the grid exploration test, there are no significant differences in left forelimb (A) and hindlimb (B) misplacements between the vehicle and DHA-treated group during the period of behavioural assessment. Results represent mean ±SEM; n=6 animals in each group.

4.4 Discussion

Previous work in our laboratory has shown the neuroprotective effect of DHA treatment in a thoracic SCI animal model (King et al. 2006; Huang et al. 2007; Lim et al. 2013). This study demonstrates for the first time that DHA has a neuroprotective effect in a cervical SCI animal model. In the histology results, we showed that DHA treatment 30 min after hemisection leads to a decreased lesion size, an increased neuronal cell number, and reduced microglial cell activation. In behavioural assessment, locomotor function and skilled forelimb movement significantly improved after DHA treatment.

4.4.1 Doses, timing and administration route of DHA after cervical SCI

For the purpose of clinical translation, a rapid delivery of neuroprotective agents is required after injury. Acute intravenous administration is adequate for this setting. The chosen timing and dose of DHA administration was based on previous studies from our laboratory showing functional improvements after SCI (King et al. 2006; Huang et al. 2007). The acute administration of DHA at a dose of 250 nmol/kg given 30 min after injury improves neurological function in a thoracic hemisection SCI. In a pilot study, no clear dose-dependent effect was seen with the bolus of DHA post-SCI, and no overt toxicity appeared up to DHA 2500 nmol/kg injected 1 hour after SCI (Huang et al. 2007). However, the beneficial effects of DHA were lost if the administration of intravenous DHA was delayed from 30 min to 3 hours after thoracic hemisection (Huang et al. 2007) or 3 weeks in my animal model (Fig 4.9). This suggests that a bolus administration of DHA only works during an early critical time window. The possible underlying mechanism will be discussed in Chapter 6.

4.4.2 Histological changes after DHA treatment

The lesion size after cervical hemisection SCI was reduced after DHA treatment. The reduction in lesion size is likely to be partly because of decreased cell death, consistent with our finding of more survival of neuronal cells and oligodendrocytes in the perilesional area following DHA treatment. Cell death after SCI involves both necrosis and apoptosis. Some studies have demonstrated that DHA reduces apoptosis after SCI (Lang-Lazdunski et al. 2003; King et al. 2006). Two main apoptosis signaling pathways have been described following SCI. One pathway is initiated by mitochondrial dysfunction, which is caused by ATP depletion, hypoxia or oxidative stress. Cytochrome c is released from mitochondria and activate caspase-9 pathway (Saikumar et al. 1998). The intrinsic pathway is mainly regulated by Bcl-2 and Bax, which are anti- and proapoptotic proteins, respectively (Saikumar et al. 1998). Of note, DHA can be catabolized to produce neuroprotective catabolites such as neuroprotectin D1 (Bazan 2005). DHA or neuroprotectin D1 can increase neuronal cell survival by upregulating the Bcl-2 family of antiapoptotic proteins, and downregulating caspase-3 and caspase-9, apoptotic proteins (Lukiw et al. 2008; Paterniti et al. 2014). Another well-established apoptosis pathway involves signaling by the TNF receptor family, including TNFR1, Fas ligand and p75. When these receptors are ligated, they recruit and proteolytically activate the initiator caspase-8 (Ashkenazi et al. 1998), which produces apoptotic cell death. These death receptors are upregulated in neurons, microglia, and oligodendrocytes after injury such as SCI (Li et al. 2000; Casha et al. 2001; Beattie et al. 2002). Several studies have demonstrated that DHA treatment can modulate the expression of TNF receptors, which may contribute to the regulation of cell apoptosis

(Moghaddami et al. 2007; Ebert et al. 2009; Paterniti et al. 2014). It can be hypothesized that DHA is able to attenuate apoptotic cell death through the intrinsic pathway.

From the current experimental data, we have found that DHA suppresses microglial activation after SCI. Macrophages express multiple phenotypes, with corresponding functions in tissue repair or damage (David et al. 2011). Activated microglia in the injured spinal cord produce various pro-inflammatory cytokines, proteases and other factors that are cytotoxic. Microglia express mRNA for IL-1β after SCI (Pineau et al. 2007). Intrathecal infusion of IL-1 receptor antagonist for 72 hours after SCI in rats markedly reduced apoptosis related to injury (Nesic et al. 2001). Furthermore, anti-inflammatory treatment with minocycline or FK506 reduced the microglial activation and lesion size after injury, and contributed to functional recovery (Stirling et al. 2004; Lopez-Vales et al. 2005). The detrimental effects of microglia may be explained by the fact that microglial activation is linked to increased expression of the inducible isoform of nitric oxide synthase (iNOS). Microglia can kill neurons through formation of superoxide and nitric oxide (Kaushal et al. 2007). It has been shown that DHA is an endogenous ligand for RXR (de Urquiza et al. 2000) and PPARs (Grygiel-Gorniak 2014). After SCI, activated microglia express the retinoic acid receptor (Mey et al. 2005). RXR agonist (9-cis-retinoic acid) has been shown to suppress the lipopolysaccharide-triggered inflammatory response of microglia and astrocytes in vitro (Xu et al. 2006). In addition, PPARγ activation can also induce apoptosis of activated macrophages (Chinetti et al. 1998) and reduce the differentiation of monocytes into macrophages (Combs et al.

2000). Thus, DHA may alleviate the inflammatory response by modulating the response of reactive microglia.

Neurofilament is present in neuronal cell bodies and axons in both phosphorylated and non-phosphorylated forms (Pant et al. 2000; Liu et al. 2004). Its phosphorylation state plays a crucial role in the regulation of its function and is vital in its degradation following CNS injury. A subunit of Phosphorylated neurofilament has used to evaluate the severity of SCI in patients (Hayakawa et al. 2012). In order to examine axonal pathology, a neurofilament marker was used (SMI31) to evaluate phosphorylated neurofilament expression following injury. In our previous study, DHA administration can significantly reduce the loss of non-phosphorylated neurofilament after compression SCI (Ward et al. 2010; Lim et al. 2013). In agreement with our previous observations, we showed here that a significant decrease in neurofilament occurred after cervical hemisection (chapter 3), and the loss of neurofilament was significantly reduced after DHA treatment in the cervical spinal cord caudal to the lesion site.

At the lesion site, although a neuroprotective effect of DHA on neuronal cells was observed, there appeared to be no obvious increase in the number of oligodendrocytes and SMI-31 positive neurofilaments in rats treated with DHA. These findings are different from observations in the thoracic SCI animal model. The reason for this discrepancy is not clear but may be due to the severity and extent of lesion (compression injury vs. hemisection injury). Our lesion size made by hemisection is smaller than the lesions made by compression or contusion injury. If the severity of the

primary injury is limited, this diminishes the extent of secondary injury in SCI. Another possible explanation is that dephosphorylated neurofilament, which was evaluated in our previous studies, is more vulnerable to injury-induced proteolysis than the phosphorylated form (Schumacher et al. 2000). DHA may exert more neuroprotection on dephosphorylated neurofilament compared to phosphorylated neurofilament.

Concerning protection of oligodendrocytes, an *in vitro* study demonstrated that DHA is necessary to protect oligodendrocytes against hydrogen peroxide-induced cell death, and neuronal glutathione activity is increased by supplementation with DHA (Brand et al. 2008). DHA treatment significantly increased oligodendrocytes survival in thoracic SCI models (Huang et al. 2007; Lim et al. 2013). However, it appears that DHA had a limited neuroprotective effect on oligodendrocytes in my animal model. One factor that should be taken into consideration is the anatomical variance between cervical spinal cord and thoracic spinal cord. The ratio between gray matter and white matter is higher in cervical spinal cord. This means that fewer oligodendrocytes were involved per cervical spinal cord cross section compared to the thoracic spinal cord, which could result in no significant effect of DHA due to the lower number of oligodendrocytes.

4.4.3 Behavioural recovery after DHA treatment

Accurate behavioral assessment is an important part of developing SCI repair strategies. Open field tests are user-friendly and widely used. In thoracic SCI, DHA treatment has been shown to improve locomotor function recovery in rodent animal models (Huang et al. 2007; Lim et al. 2013). In our open field tests, the rats treated with

DHA showed significant improvement within one week. However, the recovery of vehicle-treated rats was very comparable to that in rats with DHA treatment one week after cervical SCI. This result is different from the finding in thoracic SCI animals, in which DHA can improve hindlimb locomotor function at the end point of the experiment. The development of the BBB scoring system has enabled researchers to efficiently and reproducibly evaluate the locomotor abilities of thoracic spinal cord injured rats (Basso et al. 1996). One study reported that a hemisection of the rat spinal cord at thoracic level affected the hind limbs more severely than a cervical hemisection (Webb et al. 2002). The cervical hemisection resulted in the same BBB score as sham animals 40 days following injury. However, the BBB scores showed a significant difference between a thoracic hemisection group and the sham group. When a quantitative and more stringent analysis was used, significant alterations in the gait of rats with either a cervical or a thoracic hemisection were observed for up to 6 weeks post-surgery. This may result from the difficulty of detecting quantitative differences in a relatively non-challenging locomotor task and because the animals recover a substantial amount of their locomotor abilities following unilateral cervical hemisection. We also should keep in mind the fact that the methods used for evaluating thoracic spinal cord injured animals may not be optimal for cervical spinal injured animals.

It is not clear what the underlying changes are that produce this pattern of recovery. The recovery of locomotor ability may be due to spared pathways originating from supraspinal and propriospinal structures which can play an active role in the recovery process, and also in restoring some voluntary control (Rossignol et al. 2011). Another

possible mechanism contributing to functional recovery is the formation of new circuits. The new circuits could result from new anatomical connections (new circuits) or from enhanced connectivity (enhancing existing circuits). A more detailed discussion of this topic will be given in Chapter 5.

An attempt was made to analyze functional recovery in more detail. We employed the Montoya staircase test and grid exploration test to examine the skilled forelimb and hindlimb function on the lesion side. Notably, our results demonstrate that DHA is able to enhance skilled functional recovery. In the staircase test, in order to fully demonstrate voluntary forelimb functionality and dexterity, animals were required to grasp food pellets to eat. Improvement in both food pellets displaced and pellets eaten revealed a significant difference between the vehicle and DHA-treated groups at the end point of the test. In the grid exploration test, a significant difference in limb misplacement was observed in the control group compared with the DHA treated group throughout the behavioural testing period. As opposed to the relatively stereotyped locomotor movement, skilled locomotion requires (1) the ability to rapidly adjust muscle length and tension, (2) a high degree of intra- and interlimb coordination, and (3) the ability to voluntarily and rapidly adjust portions of the step cycle (Webb et al. 2005).

In summary, DHA treatment can significantly improve functional recovery on skilled tasks.

4.4.4 Correlation between functional outcome and histological assessment after SCI

Several studies have demonstrated a strong correlation between locomotor function and the amount of spared grey and white matter following thoracic SCI (Joshi et al. 2002; Kurita et al. 2005). In our study, we assessed the locomotion recovery after cervical SCI by using the FLS score, which has recently been shown to correlate with the extent of spared tissue at the epicenter of the lesion following cervical SCI (Singh et al. 2014). Our FLS score results demonstrating that DHA treated animals achieve better results than the vehicle group may be due to DHA producing a reduced lesion size and more neuronal cells survival. Furthermore, during the locomotor test, the neurons at C4-5 level are responsible for the “forelimb duty factor”, which represents the percentage of the total step cycle that was in stance phase (calculated as stance time / cycle time). C4/C5 right hemisection SCI rats have significantly different forelimb stride lengths and locomotor step cycle (Neckel et al. 2013). To address this issue in our animal model, further studies on detailed gait parameters would be valuable.

Another interesting histological finding after cervical hemisection is the substantially increased SMI-31 positive axons in the spinal cord caudal to the lesion site. This result implies that there is more neurofilament below the lesion site. Several studies have demonstrated that the recovery of skilled movement is positively related to the amount of axonal innervation, especially by the CST (Metz et al. 2002; Bareyre et al. 2004). The increase in axons in the DHA group suggests that DHA can either prevent progress of demyelination or promote axonal sprouting from the contralateral side. This possibility

will be explored further in the next chapter. Regarding the correlation between the number of SMI31 positive neurofilaments and the recovery of skilled forelimb task, there is no significant difference (see appendix, Figure 1).

Compared with the sham operation group, our immunohistochemical study showed that hemisection reduced 5-HT fiber intensity in ipsilateral spinal cord caudal to the lesion site. However, hemisection also resulted in an extraordinary increase in the 5-HT intensity rostral to the lesion one week after hemisection. 3 weeks after hemisection, serotonin immunoreactivity recovered to baseline levels in the caudal part of the spinal cord. A significant elevation in serotonin fibres still was found in the spinal cord rostral to the lesion site. The finding of a recovery in serotonin intensity caudal to lesion site is supported by previous investigations in thoracic spinal cord hemisection animal models (Saruhashi et al. 1996; Hains et al. 2002; Saruhashi et al. 2009)

4.5 Summary

- Our observations provide evidence that acute DHA administration has therapeutic potential in cervical SCI
- Acute DHA treatment increased the number of neuronal cells and axons in the spinal cord following cervical hemisection injury
- Acute DHA treatment reduced the microglial response after SCI
- Behavioural tests showed significantly improved skilled forelimb movement with DHA treatment

5 Effect of DHA treatment on neuroplasticity in rat cervical hemisection and mouse pyramidotomy SCI models

5.1 Introduction

It is now well established that the central nervous system is capable of substantial reorganization in cases of incomplete SCI, as cortical, subcortical and much of the local spinal cord circuitry remains intact and partially interconnected through spared axonal pathways (Raineteau et al. 2001). In view of such a potential for neuroplasticity, a variety of techniques employing pharmacological (Bradbury et al. 2002; Vavrek et al. 2006) or sensorimotor stimulation (Hoffman et al. 2007) have been developed to promote functional recovery.

DHA has been shown to induce significant functional improvements following spinal cord injury (SCI) (King et al. 2006; Huang et al. 2007; Figueroa et al. 2013; Lim et al. 2013). The studies with omega-3 fatty acids in CNS traumatic injury have focused mainly on their neuroprotective potential. However, the beneficial effects of DHA after central neurological injury could also be related to promoting neuroplasticity. Several studies have demonstrated that DHA increases maximum neurite length and the total number of neurites in embryonic hippocampal and cortical neurone cultures (Calderon et al. 2004; Cao et al. 2005), as well as in primary sensory neurons (Robson et al. 2010). In animal studies, oral DHA has been shown to promote the synthesis of components of

synaptic membranes and specific presynaptic and postsynaptic proteins (Wurtman et al. 2006) and improve brain learning involving synaptic plasticity (Wu et al. 2008). In my animals with acute DHA administration, it remains to be established whether the neurological functional recovery is due to neuroprotection only or also reflects increased axonal plasticity or synaptogenesis following DHA treatment.

In this study, the neuroplasticity effects of DHA after cervical hemisection are examined in more detail. Furthermore, in order to determine whether DHA can promote axonal sprouting in another animal species and model, we carried out pyramidotomy in mice to explore this possibility. There are several advantages to using pyramidotomy to examine structural plasticity. Compared to spinal cord lesion, a lesion of the pyramidal tract in the brainstem is more likely to spare other spinal tracts and spinal neuronal structures. In this model, we can investigate the axonal growth and the neurological functional recovery in response to a complete unilateral CST lesion. Without spinal cord tissue damage, we can avoid the confounding neuroprotection effects of DHA treatment. Any recovery of motor function following pyramidotomy is therefore likely mainly to be due to neuroplasticity.

5.2 Aims

The hypothesis in this chapter is that DHA can improve neurological recovery by promoting axonal sprouting and synaptic function. The aim of the study presented in this chapter was to assess the neuroplasticity effect of an acute i.v. administration of DHA in (1) rat cervical hemisection and (2) mouse pyramidotomy. In both models, we studied

the effect of DHA treatment on skilled motor function and also on tissue neuroplasticity markers.

5.3 Results

5.3.1 DHA treatment increases synaptic terminals in the spinal cord caudal to lesion site after SCI

Synaptophysin, a synaptic vesicle protein in presynaptic axons, represents a reliable marker for the quantification of synapses in CNS tissue (Calhoun et al. 1996) and has been used previously as a marker to show modification in neuronal circuitry (Mitsui et al. 2005). Synaptophysin is known to play an essential role in activity-dependent synapse formation (Spiwoks-Becker et al. 2001; Tarsa et al. 2002). Synaptophysin immunoreactive boutons provide an efficient way to visualize synaptic profiles within the CNS, and synaptophysin immunostaining has been used widely to estimate an increase or decrease in synaptic contacts at light microscopic level (Toggas et al. 1996; Jeffrey et al. 2000; Zang et al. 2005). Synaptic terminals were examined by synaptophysin immunostaining 3 weeks after SCI. In the cervical spinal cord caudal to the lesion site, synaptophysin immunoreactive terminals were significantly increased in the DHA treatment group compared to the vehicle group (at 1250 μm caudal to lesion site (Fig 5.1). We also used Western blotting to quantify the synaptophysin protein levels in the cervical spinal cord 3 weeks following injury in order to verify our immunostaining findings (Fig 5.2).

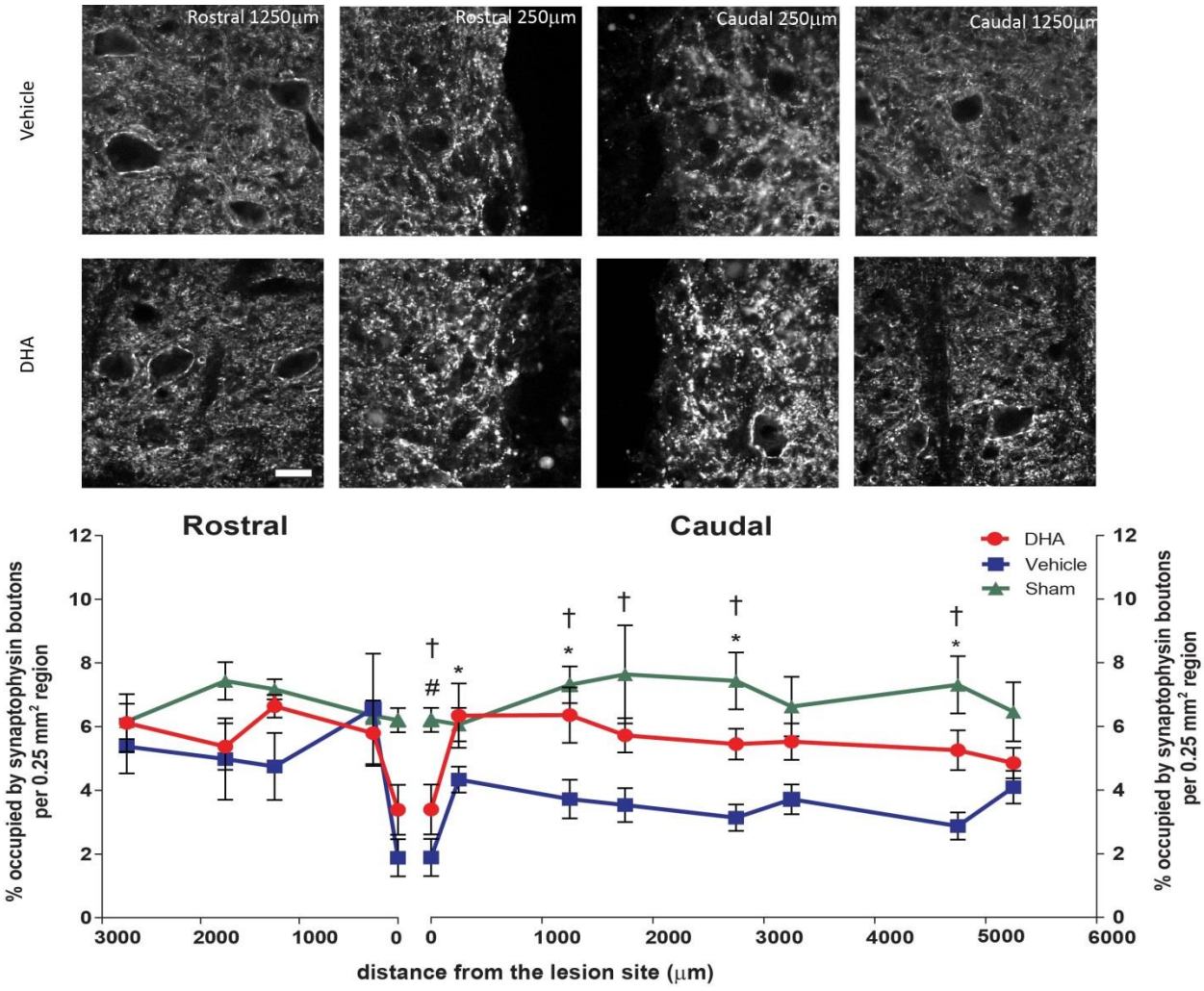

Figure 5-1 Effect of DHA treatment on synaptic terminals after cervical hemisection in the rat

Photomicrographs showing immunocytochemical labeling of synaptophysin in different regions of longitudinal sections of the cervical segment of the spinal cord following hemisection. Scale bar =100 µm. Analysis of synaptophysin immunoreactivity revealed significantly more synaptic terminals in the DHA treated group (red circles) and sham operated group (green triangles) compared to the vehicle treated group (blue squares) caudal to the lesion site. * P<0.05 DHA vs. vehicle group, † P<0.05 sham vs. vehicle group. # P<0.05 sham vs. DHA group. Results represent mean ±SEM. n=6 animals per group.

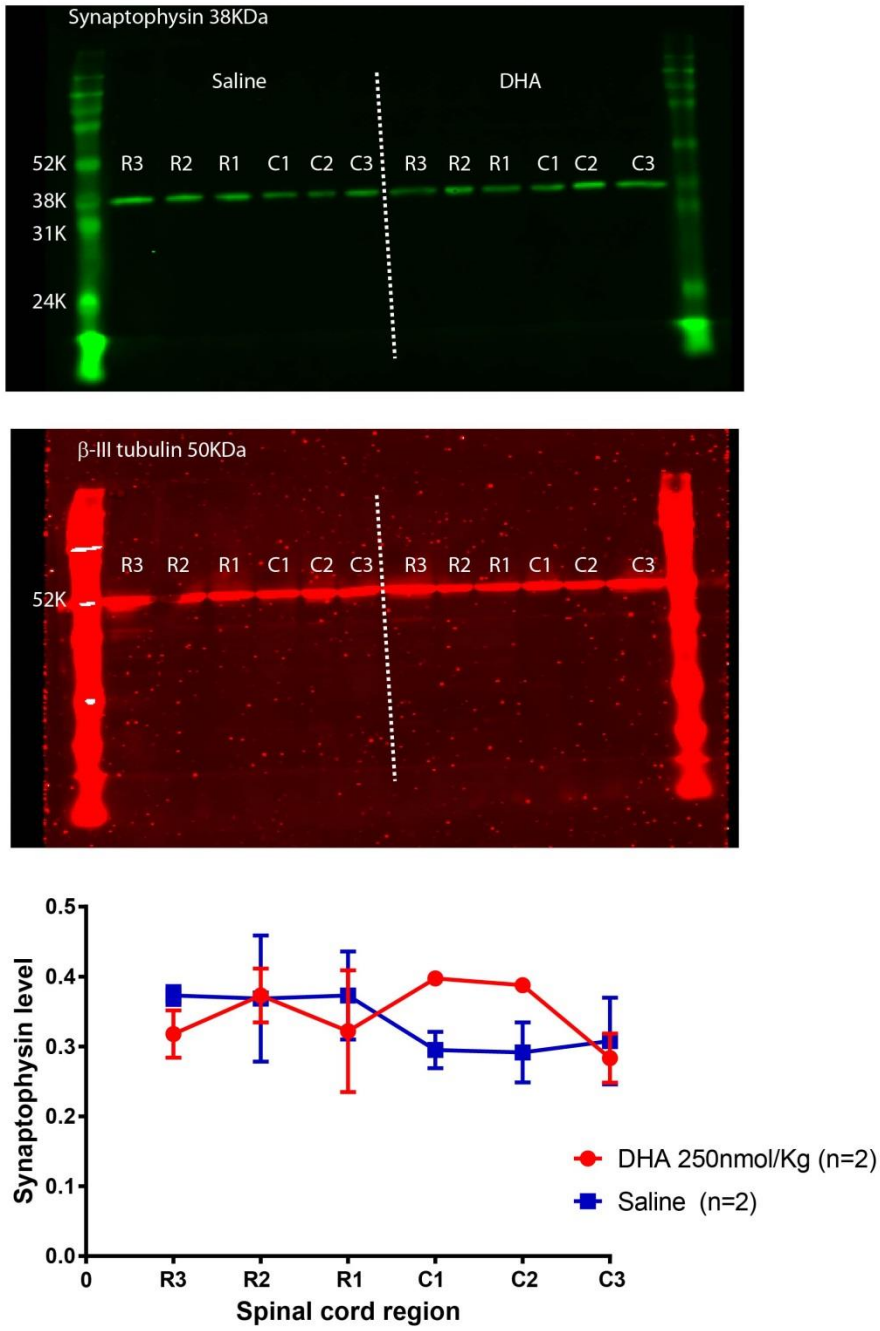

Figure 5-2 DHA increases synaptophysin in the spinal cord caudal to the lesion site.

Western blot analysis using β3-tubulin as standard control showed that DHA increases synaptophysin protein in the spinal cord 0-3 mm, 3-6 mm caudal to the lesion site. R3 represents 6-9 mm rostral to lesion site. R2 represents 3-6 mm rostral to lesion site. R1 represents 0-3 mm rostral to lesion site. C1 represents 0-3 mm caudal to lesion site. C2 represents 3-6 mm caudal to lesion site. C3 represents 6-9 mm caudal to lesion site. Results represent mean ±SEM; n=2 animals in each group.

5.3.2 DHA increases synaptic boutons contacting motor neurons in the cervical spinal cord

Goat anti-Choline Acetyltransferase (ChAT) antibody was used to localize the motor neurons in ventral horn 5 mm caudal to lesion site that affect forelimb motor function after SCI. Further analysis revealed that DHA treatment increased the amount of synaptophysin protein in boutons around motor neurons in the cervical spinal cord ventral horn 2mm caudal to lesion site after hemisection (Fig 5.3), which could correlate with neurological functional recovery.

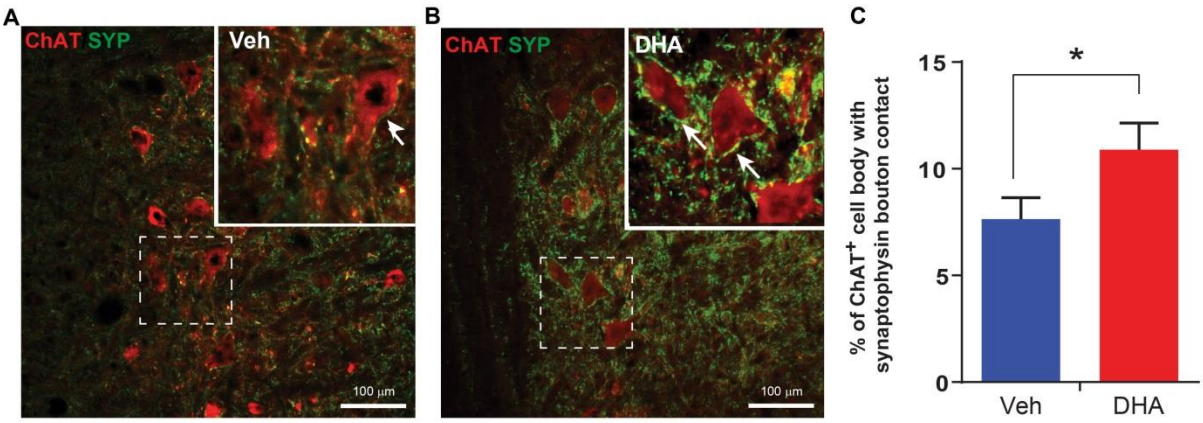

Figure 5-3 Effect of DHA on synaptic boutons contacting motor neurons

(A,B) Images showing the difference in the density of contacts between synaptophysin terminals and ChAT motor neurons in DHA and vehicle groups. Scale bar =100 μm **(C)** Quantitative analysis confirmed the increase in density of synaptophysin contacts on ventral horn motor neurons in DHA treated rats. * $P<0.05$ DHA vs. vehicle group. Results represent mean ±SEM; n=6 animals in each group.

5.3.3 DHA treatment increases serotonin fibres ipsilateral to the lesion

The spinal serotonergic pathway plays an essential role in modulating and triggering neuronal activity in the spinal cord (Ciranna 2006; Jordan et al. 2008). Quantification of cervical spinal cord serotonin immunoreactivity in the DHA treatment group and the vehicle group 21 days following cervical hemisection is shown (Fig 5.4). A dramatic increase in serotonin axons was observed in the spinal cord rostral to the lesion site in both groups. There was a significant difference between the two groups caudally. In the DHA treatment group, serotonin axons were more abundant than in the control group in the caudal part of the spinal cord.

5.3.4 DHA increases serotonin contacts with motor neurons

In addition to measuring the global immunoreactivity through the whole cervical spinal cord in longitudinal sections, I also assessed the density of 5-HT containing terminals contacting individual motor neurons, in order to provide more information on a specific site related to motor function recovery.

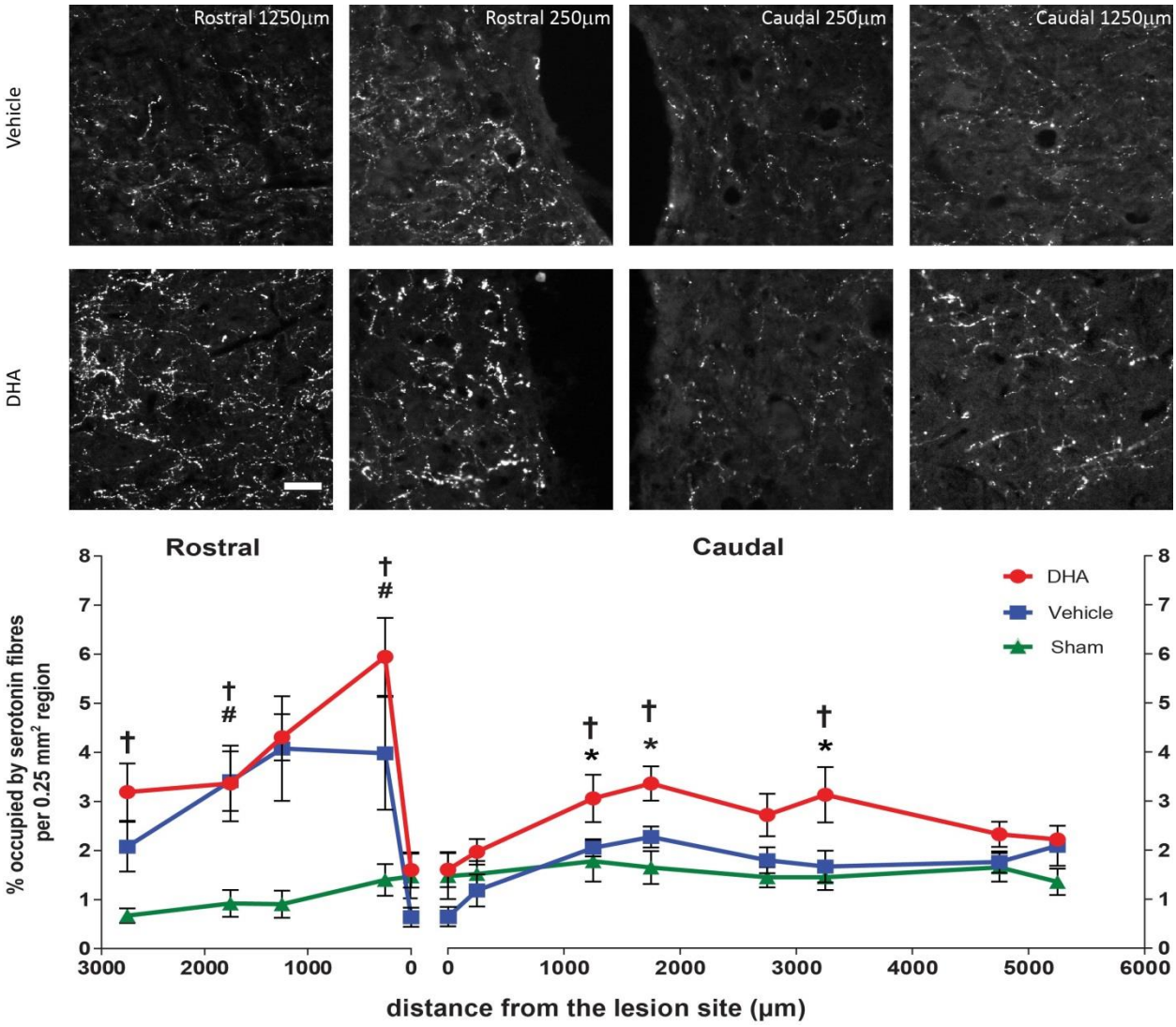

Figure 5-4 Effect of DHA treatment on serotonin terminals

Photomicrographs of serotonin immunofluorescence in representative sections of a vehicle and DHA treatment group. Compared to the vehicle group, a higher staining intensity was observed. The area occupied by serotonin axons was quantified in both groups. Scale bar =100 µm. The data revealed that serotonin fibres both rostral and caudal to the lesion site were significantly increased in the DHA treatment group compared to the vehicle group (*P<0.05). * P<0.05 DHA vs. vehicle group, † P<0.05 sham vs. vehicle group. # P<0.05 sham vs. DHA group. Results represent mean ±SEM. n=6 animals per group.

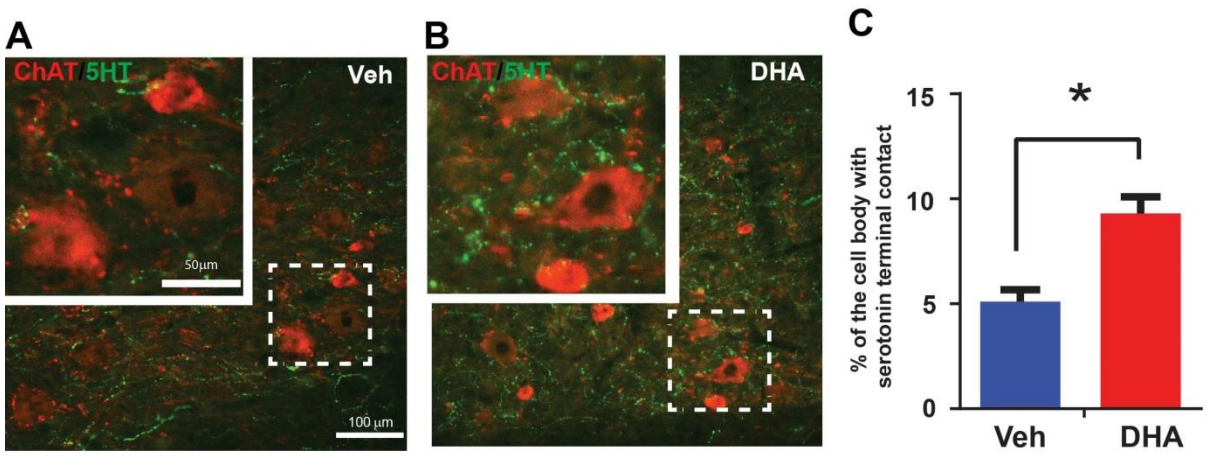

Figure 5-5 DHA enhances serotonin fibres surrounding motoneurons

(A,B) Images show the difference in density of contacts between serotonin (5-HT) immunopositive terminals (green) and choline acetyltransferase immunopositive motor neurons (red) in DHA- and vehicle-treated groups. Scale bar =100 µm. **(C)** Quantitative analysis confirmed the increase in density of serotonin contacts on ventral horn motor neurons in DHA treated rats (red bar) compared to vehicle treated animals (blue bar). * $P<0.05$ DHA vs. the vehicle group. Results represent mean ±SEM. n=6 animals per group

5.3.5 DHA treatment increases sprouting of CST axons at a lesion site

BDA was unilaterally injected into the ipsilateral hemisphere to anterogradely label intact CST axons and collaterals in rats following cervical spinal hemisection (Fig.5.6). Sprouting fibres in the grey matter on the lesioned side could be detected by immunofluorescence. DHA treated animals had increased CST collateral sprouting on the lesion side, which was significantly higher at the spinal cord level 1 mm caudal to the lesion site. In contrast, the vehicle group had only a few BDA-labelled CST axons in the grey matter of the lesioned side (Fig. 5.6). DHA treated animals also had increased CST axons on the unlesioned side (Fig. 5.6).

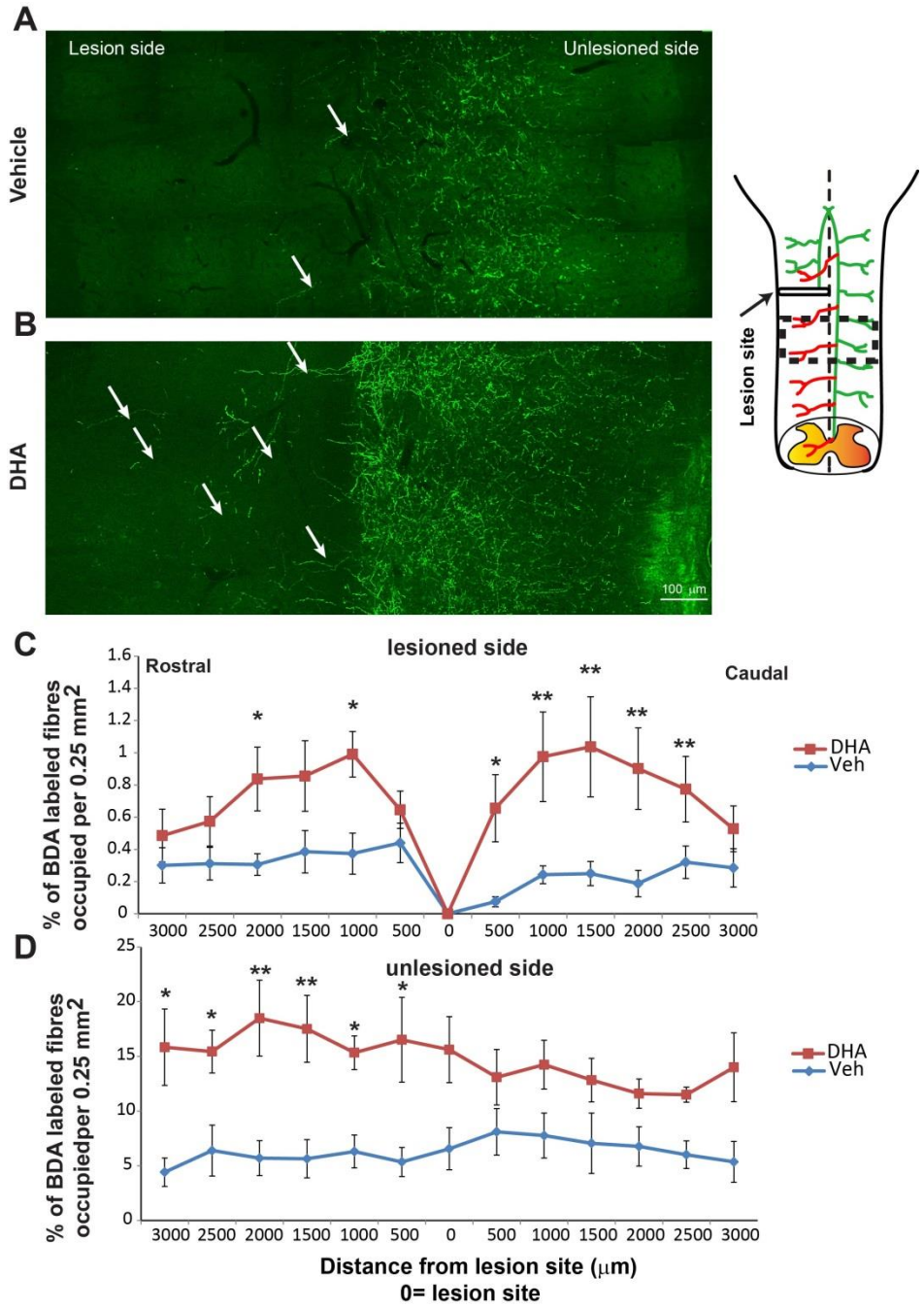

Figure 5-6 Effect of DHA treatment on corticospinal axons sprouting in cervical hemisection rats.

(A,B) Photomicrographs of horizontal sections 1500 μm caudal to the lesion site reveal more sprouting of CST axons across the midline to the lesion side in the DHA treatment group. Scale bare =100 μm. (C,D) Quantification of labelled CST fibres after spinal cord lesion showed a significant increase in the spinal cord caudal and rostral to the lesion site on the lesion side (C) and increase in the spinal cord rostral to the lesioned site on unlesioned site (D). * P<0.05, ** p< 0.01 DHA vs. the vehicle group. Results represent mean ±SEM. n=5-6 animals per group

5.3.6 Effect of DHA in unilateral pyramidotomy

In order to determinate whether DHA can promote axonal sprouting in another animal species and model, BDA was injected into the contralateral hemisphere in mice receiving a right unilateral pyramidotomy (Fig.*5.7*). Adult mice with a unilateral pyramidotomy were injected with a single bolus of DHA (500 nmol/kg) or vehicle (saline with 0.2% ethanol) 30 min after injury. A higher dose of 500 nmol/kg was used in mice compared to 250 nmol/kg used in rats based on our previous published data due to species and metabolic differences (Lim et al. 2013).

5.3.7 DHA treatment increases sprouting axons

BDA was injected in the sensory-motor cortex ipsilateral to lesion site four weeks following pyramidotomy. A significant increase in the number of sprouting axons was detected in the DHA treatment group compared to the vehicle group (Fig. 5.8)

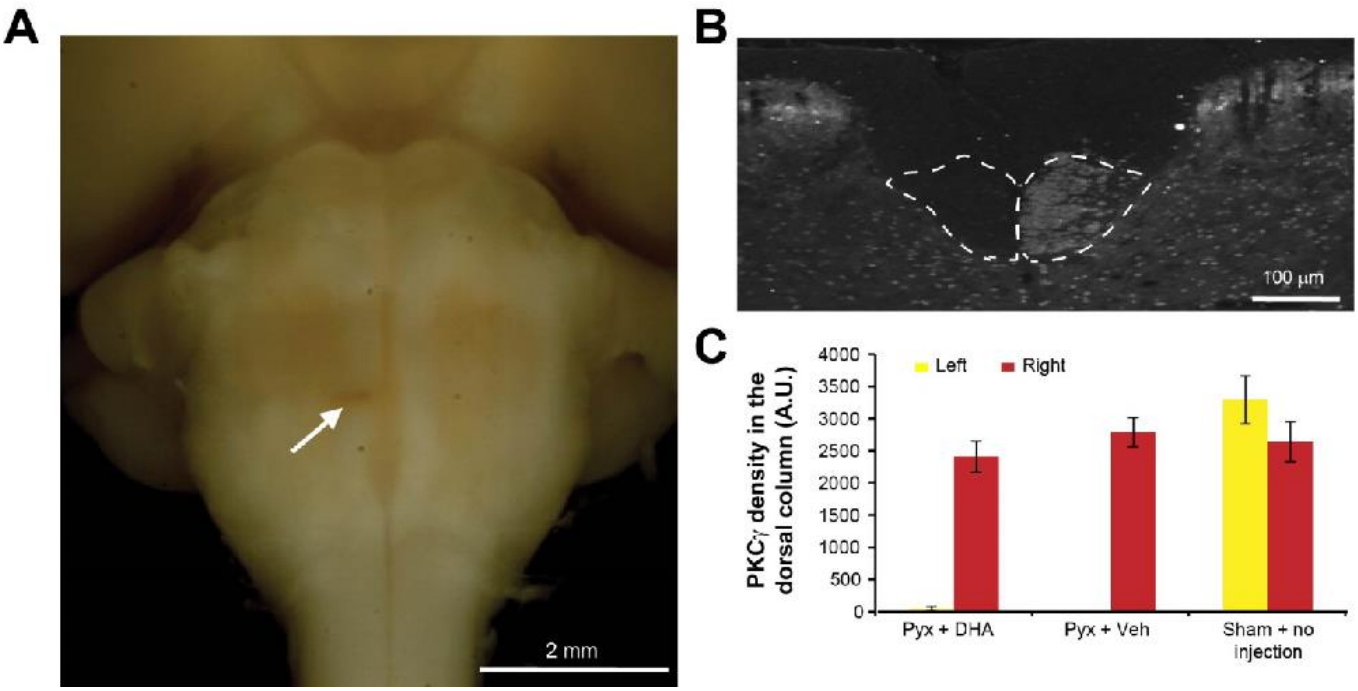

Figure 5-7 Pyramidotomy in mice

(A) Ventral view of the brainstem of mice following pyramidotomy, showing a unilateral lesion of the CST running in the medullary pyramids. The arrow indicates the transection site in the right pyramidal tract. **(B)** Loss of PKCγ immunostaining in the main portion of the tract in the dorsal funiculus of the cervical spinal cord confirms lesion of the CST. Scale bare =100 μm. (**C)** The intensity of PKCγ immunostaining of the bilateral CST in the cervical spinal cord revealed a significant decrease on the lesion side (left) after pyramidotomy compared to the sham operation group. However, there was no significant difference in PKCγ staining between the DHA and vehicle group. Results represent mean ±SEM; n=6 animals in each group.

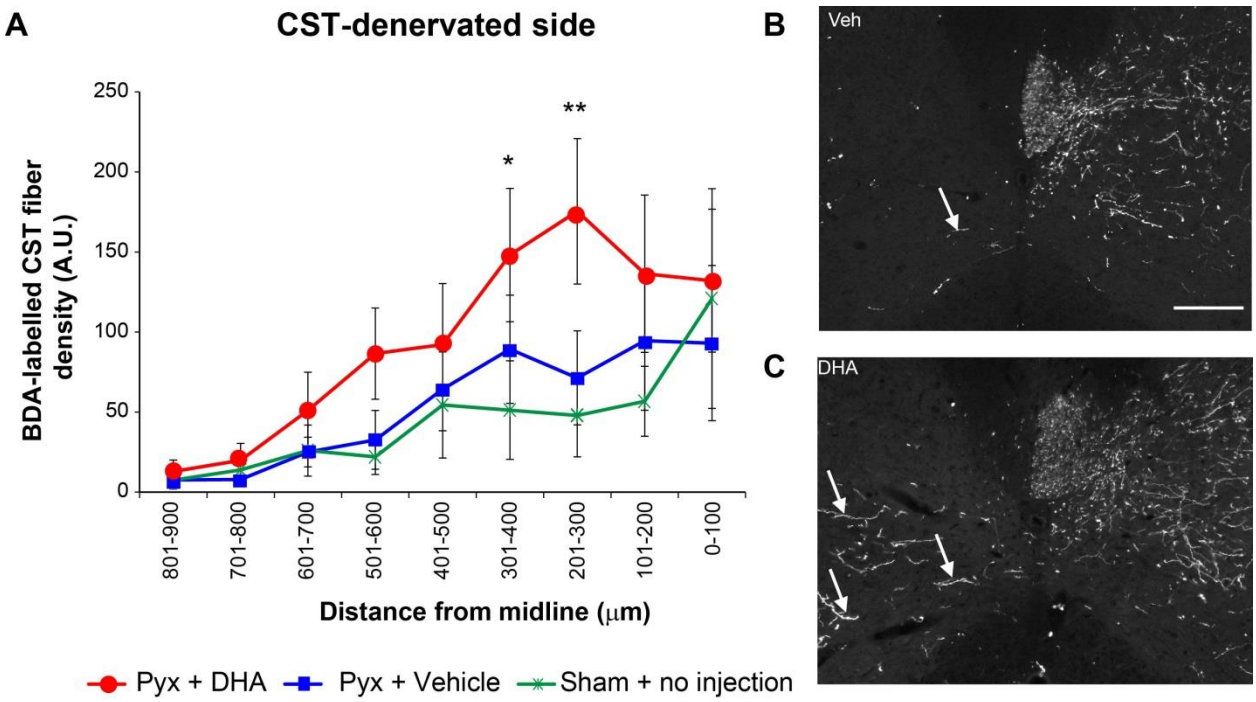

Figure 5-8 DHA treatment increases CST axonal sprouting in mice following pyramidotomy

Transverse sections of the cervical spinal cord reveal CST fibres sprouting from the contralateral side after pyramidotomy in both the vehicle **(B)** and DHA **(C)** treated groups. Scale bar =100 μm. **(A)** Quantitative analysis of midline crossing fibres showed that the DHA-treated group had significantly more midline crossing fibres in the CST-denervated side of the cervical spinal cord compared to vehicle and sham groups. Results represent mean ±SEM; n=4-5 animals in each group.

5.3.8 DHA treatment improved skilled locomotor activity

In addition to the analysis of CST sprouting fibres, a behavioural test was performed to determinate if the increased sprouting fibres are associated with functional recovery. During the 4-week period after CST injury, the grid exploration test was used to assess sensorimotor function in vehicle and DHA treated mice. No difference was detected between the groups in forelimb grid exploration in the first week (Fig. 5.9 A). The poor performance of the affected forelimb is likely due to failure of CST re-innervation in both groups. However, 2 weeks after injury, DHA-treated mice had fewer misplacements compared with the vehicle group. A significant difference between the two groups was found 3 weeks after injury. In the hindlimb grid exploration test (Fig. 5.9 *B*), fewer misplacements were observed in the DHA treated group compared with the vehicle group throughout the behavioural testing period.

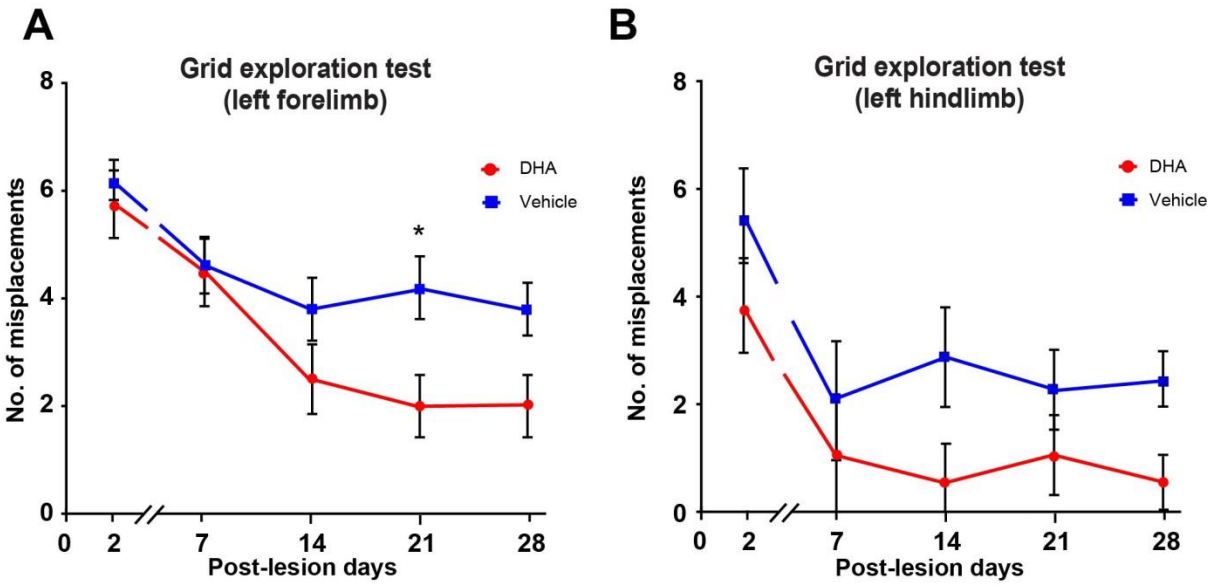

Figure 5-9 Effect of DHA on behavioural recovery in mice following pyramidotomy

(A) Mice failed to accurately grasp the rungs of the grid with the left (lesioned CST) forepaw following pyramidotomy in both groups in the first week. However, the DHA treated mice made significant fewer errors 3 weeks following pyramidotomy. (* P< 0.05) **(B)** Quantification reveals that DHA treated mice made fewer footslip errors with their left hindpaw than vehicle group animals throughout the testing period. Results represent mean ±SEM. n=5 animals per group.

5.3.9 Sprouting CST axons contact interneurons

A major question raised by the findings that DHA promoted sprouting of CST fibres is the functional relevance of these anatomical changes. According to a previous study, sprouting CST fibres may contact propriospinal interneurons following SCI. These new contacts are maintained on propriospinal interneurons that project past the lesion and form a circuit that allows transmission of descending signals below the lesion (Bareyre et al. 2004; Flynn et al. 2011). Recently, a group of ventrally located neurons was designated V2a interneurons. This group of interneurons play a key role in maintaining locomotor rhythmicity and in ensuring appropriate left–right alternation and firing of ipsilateral motor neurons during locomotion (Cowley et al. 2008; Dougherty et al. 2010). The V2a interneurons constitute a subpopulation of the V2 class and express Chx10. The Chx10 neurons are exclusively glutamatergic and project ipsilaterally (Al-Mosawie et al. 2007). However, previous studies did not provide any information about the relationship between the innervation of the Chx10 population and the recovery of skilled locomotor function after SCI. Using immunohistochemistry, V2a interneurons in the spinal cord could be identified using a Chx10 antibody (Fig 5.10). Double immunolabelling revealed that sprouting CST fibres appeared to contact V2a interneurons. Confocal analysis of selected sections confirmed that some CST fibres made contacts with Chx10 immunoreactive cell bodies, revealed using blue fluorescent Nissl staining (Fig 5.11).

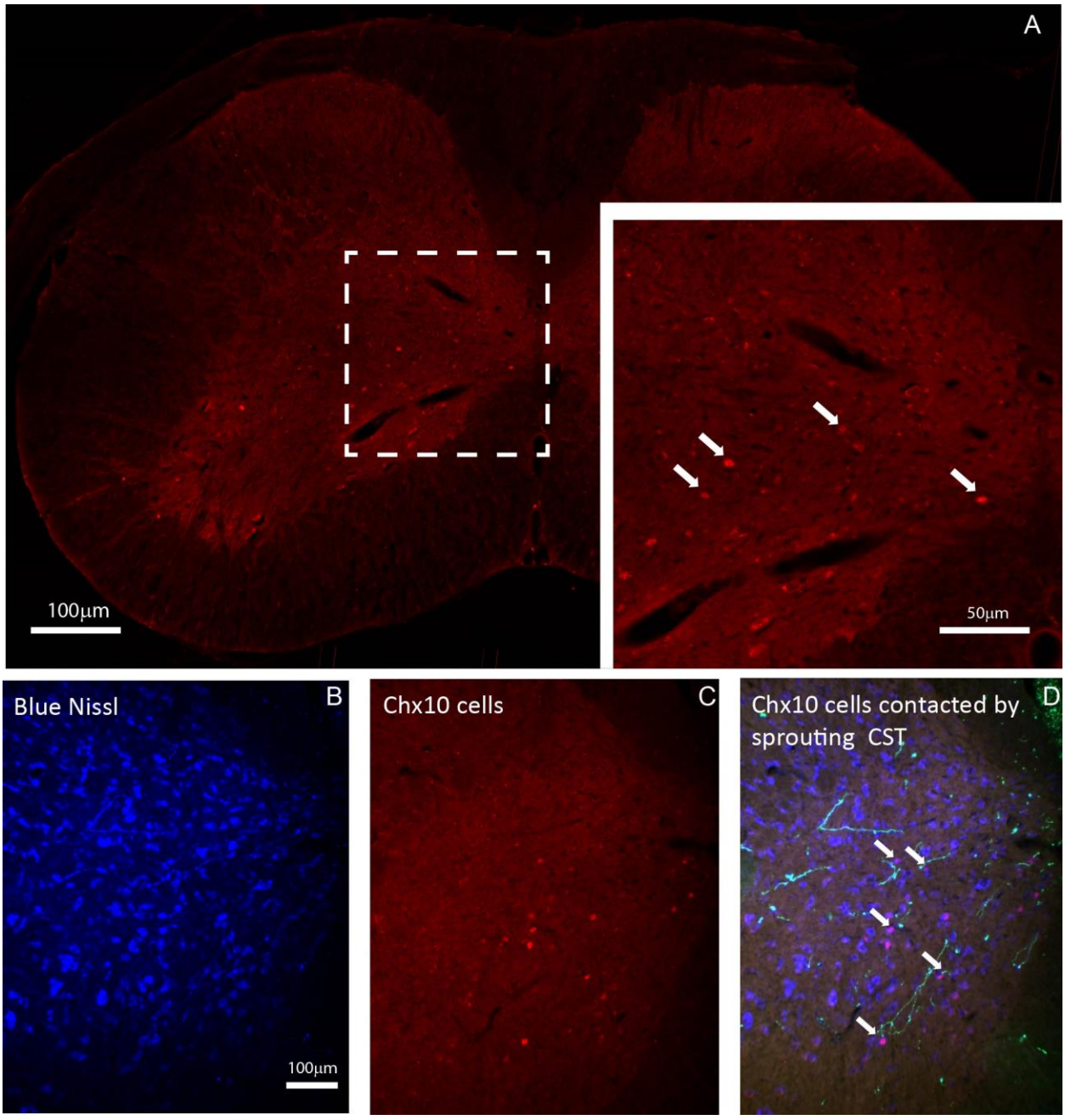

Figure 5-10 V2a interneurons express the transcription factor Chx10.

(A) A transverse section of mouse cervical spinal cord showing Chx10 positive cells located in lamina X. Chx10 immunoreactivity is present in the nuclei of V2a-derived interneurons (arrows in the inset). **(B)** Blue Nissl staining labelled all neurons in the spinal cord. **(C-D)** With BDA labelling, some Chx10 cells were seen to be contacted by CST axons (arrows).

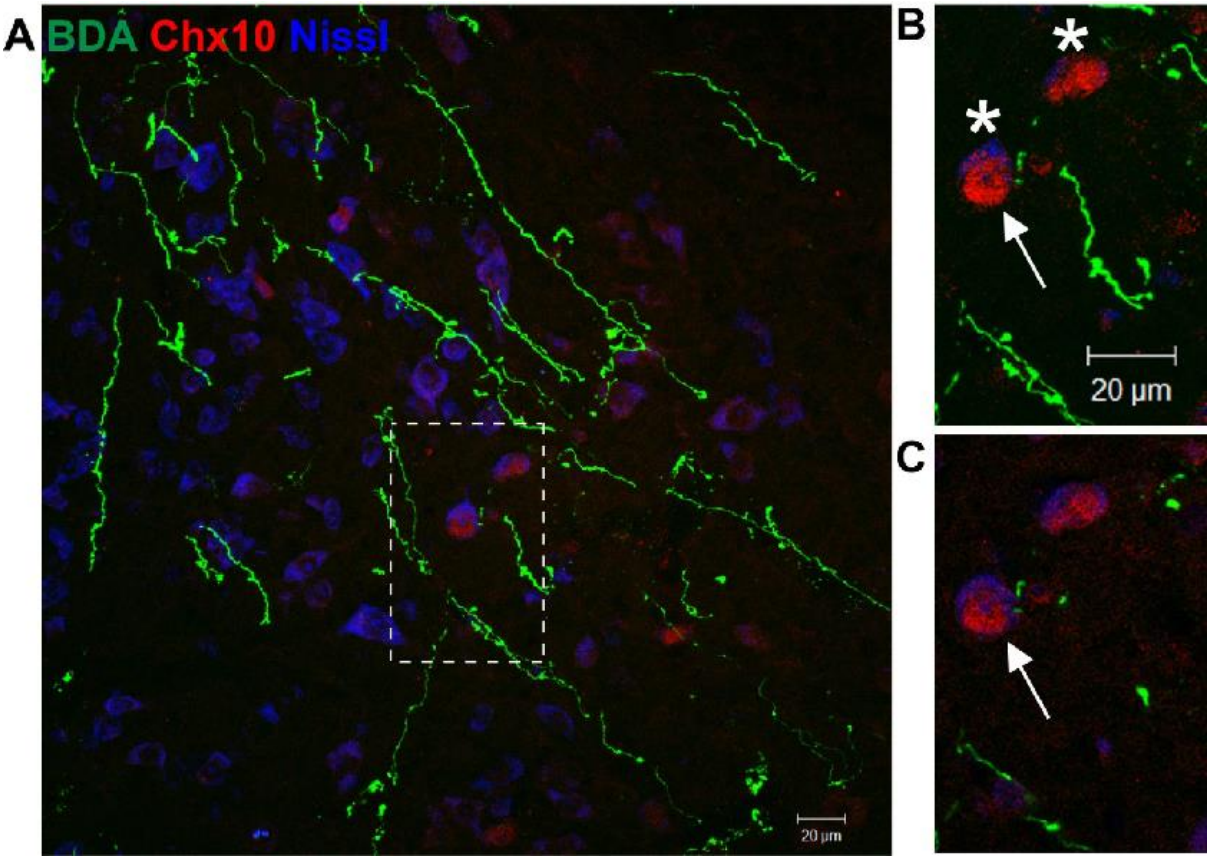

Figure 5-11 Confocal images of mouse cervical spinal cord transverse sections

Representative images showing examples of BDA-labelled CST collaterals (green) in the vicinity of blue fluorescent Nissl stained cells (Neurotrace 435/455), some of which are V2a interneurons identified by immunostaining for Chx10 (red). The dashed boxes in panels A are shown at higher magnification in B and C, which reveal contacts (arrows) between BDA-labelled collaterals and Chx10 interneurons (asterisks). **(A)** A Z-stack comprising 10x 0.66 µm optical images. **(B)** A Z-stack, comprising 25 x 0.72 µm optical images) and **(C)** a single 0.72 µm optical image.

5.3.10 DHA treatment increased the number of interneurons contacted by sprouting CST fibres

In order to identify possible targets of the CST sprouting and circuitry that could contribute to locomotion recovery, we carried out specific staining for V2a interneurons, which are responsible for locomotion control and coordination in mice (Al-Mosawie et al. 2007; Dougherty et al. 2010). Immunostaining of Chx10 labelled V2a interneurons in spinal cord transverse sections revealed that BDA-labelled sprouting CST fibres contact more V2a interneurons in the DHA treatment group than in saline controls (Fig.5.12 A). In addition, we also examined the correlation between the number of Chx10 cells contacted by CST axons and forelimb misplacement. We found that higher numbers of Chx10 cells contacted by CST axons correlated with less forelimb misplacement (Fig 5.12 B).

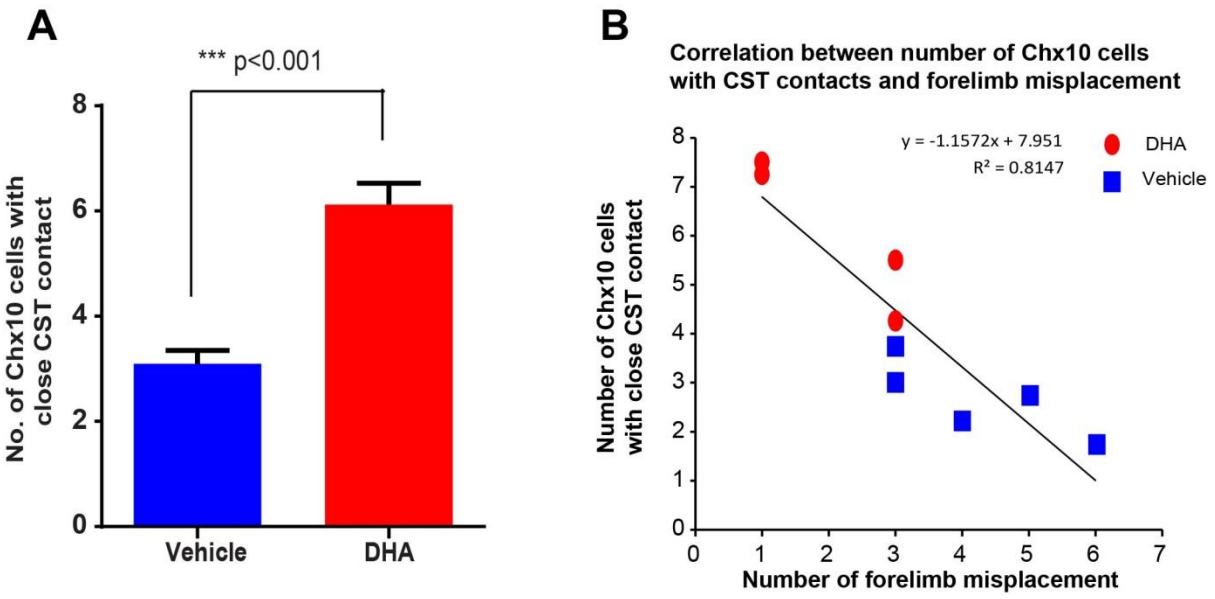

Figure 5-12 DHA treatment increases the number of interneurons contacted by sprouting CST axons

(A) Analysis revealed a significant increase in the number of Chx10 interneurons contacted by BDA-labelled CST collaterals following DHA treatment (red bar) compared to vehicle treatment (blue bar). **(B)** A strong negative correlation was observed between the numbers of Chx10 interneurons with BDA-labelled CST contacts and the numbers of forelimb misplacements. Data was taken from DHA treated (red circles) and vehicle treated animals (blue squares). *** P<0.001 DHA vs. vehicle group. Results represent mean ±SEM; n=5-6 animals in each group

5.4 Discussion

To date, several DHA studies in different SCI models have shown significant neurological recovery which appears to be due to a neuroprotective effect (King et al. 2006; Huang et al. 2007; Lim et al. 2013; Paterniti et al. 2014). However, the effect of DHA on neuroplasticity has not been investigated in SCI. This present study demonstrates that DHA can induce neuroplasticity after SCI by promoting sprouting of axons. Functional and anatomical plasticity was demonstrated by the behavioural and histological assessment in two different rodent species and models. Furthermore, studies of cervical hemisection show that DHA not only induces axonal sprouting but also exerts a positive effect on synaptogenesis after SCI.

5.4.1 DHA enhances synaptogenesis

In the current study, we address the question whether the acute administration of DHA can enhance synaptogenesis following CNS injury. In the cervical hemisection animal model, our results indicate that the level of synaptophysin increased in the spinal cord ventral horn caudal to the injury site in the DHA group. The elevation in synaptophysin levels implies that upregulation of synaptic function occurs after treatment, which is related to functional improvement. This finding is consisted with other experimental studies. In rats and mice, individual differences in spatial learning capacity are correlated with the level of synaptophysin expression in the hippocampus (Smith et al. 2000; Frick et al. 2003). In another experimental study, the neurological performance in rats receiving motor training is proportional to the extent of synaptophysin in thalamus (Ding et al. 2002). In the spinal ventral horn, the changes in synaptophysin expression

could modulate motor function in a mouse model of amyotrophic lateral sclerosis (ALS) (Zang et al. 2005) and in a traumatic SCI animal model (Macias et al. 2009).

In an effort to gain more insight into synapse formation on appropriate caudal targets, such as the motor neurons that are responsible for specific forelimb movements, synaptophysin expression surrounding motor neurons was measured. The data revealed that synaptophsin was significantly augmented in the region of motor neurons.

Our findings are similar to other animal models treated with DHA. Oral DHA has also been shown to promote the synthesis of synaptic membranes and specific presynaptic proteins (synapsin-1 and syntaxin-3) and postsynaptic proteins (postsynaptic density protein 95) (Wurtman et al. 2006; Cansev et al. 2007). Upregulation of these synaptic proteins after dietary consumption of DHA is also linked to improvement in brain learning (Wu et al. 2008) and spinal cord sensorimotor learning following SCI (Joseph et al. 2012).

Previous studies demonstrated that synaptophysin immunoreactivity is reduced in the spinal cord caudal to the lesion site after spinal cord hemisection or transection (Nacimiento et al. 1995; Macias et al. 2009; Lopez-Dolado et al. 2013), which is compatible with our results. In our study, the global synaptophysin expression decreased below the lesion site. However, the synaptophysin immunoreactivity in the DHA treated group was significantly higher than in the vehicle group. We also quantified the synaptophysin boutons contacting motor neurons in the cervical spinal cord and this demonstrated an enhancement of synaptic input to motor neurons after DHA treatment.

This study thus provides some insight into changes in synaptic input to motor neurons that affect forelimb motor function after cervical spinal hemisection.

The enhanced synaptic input to motor neurons treated with DHA suggests an essential role of DHA in promoting synapse formation and/or pre-synaptic neurotransmitter release. The observed increases in pre-synaptic proteins may involve activation of transcriptional factors following injury. DHA has been shown to be an endogenous ligand for retinoid X receptor (de Urquiza et al. 2000). SCI is associated with changes in retinoid signalling (Schrage et al. 2006), which is involved in the control of synaptic plasticity, cytoskeleton, signal transduction and ion channel formation during neurodevelopment (Maden 2002; Lane et al. 2005). One possible mechanism is that DHA binding to retinoid X receptor enhances its transcriptional activity, which upregulates synaptic activity.

In the CNS, neurotransmitter release is activated by pairing a synaptic vesicle-SNARE (soluble N-ethylmaleimide-sensitive fusion (NSF) protein attachment protein receptor) complex with its target traffic-SNARE on the intracellular surface of the plasma membrane (Jahn et al. 2006). Concerning changes in synaptic transmission following DHA treatment, DHA can influence SNARE protein expression or ternary complexes, and increase co-localization and interaction of SNAP-25 and syntaxin-3, and upregulate synaptic membrane biogenesis (Darios et al. 2006; Pongrac et al. 2007; Mazelova et al. 2009). A set of experimental data demonstrates that DHA can induce modifications in several synaptic neurotransmission systems. DHA can promote glutamatergic synaptic activity by increasing glutamate receptor subunits (NMDA receptor subunit 2B (NR2B) and glutamate receptor 1 (GluR1)) in hippocampal neurons (Kim et al. 2011).

Conversely, deficiency of DHA results in decreases in glutamate receptor units (GluR1, GluR2, NR1, NR2A, and NR2B), which leads to impairment in long-term potentiation in the hippocampus (Cao et al. 2009). Omega-3 PUFA deficiency can also alter monoamine systems. Omega-3 PUFA deficiency results in changes in the vesicular pool of serotonin as well as dopamine, leading to irreversible alteration in specific brain functions (Chalon 2006). Mice on a DHA deficient diet show augmented amphetamine-induced locomotor sensitization, associated with an alteration in the mesolimbic dopamine pathway (McNamara et al. 2008). DHA deficiency also causes a 10% cholinergic transmitter reduction in muscarinic receptor binding, although acetylcholinesterase activity and the vesicular acetylcholine transporter were not affected (Aid et al. 2003).

5.4.2 DHA induced axon plasticity

SCI causes an immediate paralysis of muscles innervated by motor neurons caudal to the lesion site. This insult is partly due to loss of cortical axons that control voluntary limb movements, using glutamate (Jordan et al. 2008). Another cause is the loss of brainstem-derived axons that provide motor neurons with a source of neuromodulators, particularly serotonin (Rekling et al. 2000; Jacobs et al. 2002). It is known that gross motor recovery depends on various sensory-motor tracts sprouting or regenerating (Bareyre et al. 2004; Bradbury et al. 2006). This axonal growth may involve spared axons, collateral sprouting, and/or changes in local spinal circuitry (Bradbury et al. 2006). In this chapter, we evaluated two different descending axonal systems (the

serotonergic system and the CST pathway) to determine if DHA has an effect on promoting axon sprouting which could be responsible for functional recovery.

5.4.2.1 The serotonergic system

Serotonergic pathways arise from medullary raphe nuclei, then they mainly pass through the ventrolateral funiculus and ventral funiculus and terminate within the ventral horn, innervating motor neurons and interneurons (Sharma et al. 1997; Gerin et al. 2010). Previous studies demonstrated that rapidly diminished spinal levels of serotonin occur ipsilateral to spinal lesion following thoracic SCI, with levels of serotonin returning by 4 weeks after injury in the lumbar region (Saruhashi et al. 1996; Hains et al. 2002; Saruhashi et al. 2009). It has been reported that increased sprouting of serotonergic fibres in the injured spinal cord leads to improved recovery of locomotion (Li et al. 2005). In our study, DHA treatment also remarkably enhanced axonal sprouting in the spinal serotonergic pathway, which plays an essential role in modulating and triggering neuronal activity in the spinal cord (Ciranna 2006; Jordan et al. 2008).

We evaluated 5-HT immunoreactive axons by two methods. The first method was to measure the global immunoreactivity through the whole cervical spinal cord in longitudinal sections. Several SCI studies have demonstrated that locomotor activity is modulated and triggered by serotonin and serotonin precursors or agonists, and that enhanced motor performance can result from stimulation of serotonin receptor subtypes (Cowley et al. 1994; Kiehn et al. 1996; Ung et al. 2008). To address this problem, we assessed the density of 5-HT containing terminals contacting individual motor neurons in the ventral horn, to provide more information on a specific site related to motor function recovery. With respect to the changes in the expression of serotonin, it is

notable that the serotonin level not only increased in the perilesional area in the DHA treatment group, especially in the spinal cord caudal to the lesion site. We also found the level of serotonin contacting motoneurons significantly increased. The present results of the serotonergic innervation of the ventral grey matter suggest that DHA stimulates axonal plasticity within the spinal neuronal network, which may eventually result in enhanced locomotor performance.

Previous studies have shown that DHA supplementation influences serotonin level and serotonin receptors in the CNS. A positive association has been reported between the amount of dietary DHA and brain 5-HT in piglets (de la Presa Owens et al. 1999). Conversely, rats with a chronic omega-3 polyunsaturated fatty acid dietary deficiency have a low response to fenfluramine-induced 5-HT stimulation (Kodas et al. 2004). In addition, omega-3 polyunsaturated fatty acid supplementation reverses the stress-induced reduction in serotonin levels (Vancassel et al. 2008). Recently, one study showed that a high-saturated-fat diet increases 5-HT_{1A} and 5-HT_{2A} receptor binding densities in the brain, which leads to cognitive dysfunction in rats. DHA supplementation can reverse changes in 5-HT receptors and thus attenuate such alteration (Yu et al. 2013). In our data, the acute administration of DHA appears to upregulate 5-HT fibres following SCI, which may contribute to motor function recovery.

5.4.2.2 Corticospinal tract axons

The CST is a major descending pathway contributing to the control of voluntary movement in mammals. About 95% of all corticospinal axons in rat are located in the ventral aspect of the dorsal columns and about 3–5% in the medial aspect of the ventral columns (Brosamle et al. 1997). Limited spontaneous sprouting of lesioned CST fibers

has been reported at the site of a spinal cord lesion in adult rats (Aoki et al. 1986; Li et al. 1994; Weidner et al. 2001). One study confirmed that the spontaneous sprouting of CST fibres contributes to locomotor recovery, by carrying out electrophysiological assessment (Bareyre et al. 2004).

In our cervical hemisection animal model, DHA treatment significantly increased the axonal density in the main CST projections contralateral to the hemisection lesion, as well as causing sprouting of CST fibres within the grey matter in the ipsilateral regions rostral and caudal to the lesion site. This finding of an increase in axonal density in the main CST and collateral sprouting fibres may be related to the ability of DHA to promote axonal plasticity. In our behavioural assessments, we obtained evidence for a functional role of this DHA-promoted CST sprouting by demonstrating a precise and positive correlation with improved food pellet grasping, a task known to be associated with CST function (Kanagal et al. 2009).

Our findings are in agreement with previous study results, which have shown that skilled movement recovery is closely linked to CST sprouting. To investigate the axonal sprouting after injury with/without treatment, several animal models have been used to discover the underlying mechanism, such as bilateral dorsal column crush, bilateral dorso-lateral column ablation, partial and complete pyramdiotomy. The lesion created by cervical hemisection included a substantial extent of grey matter. The lesion area included premotor interneurons, propriospinal neurons and possibly, motor neurons that form the segmental circuitry underlying forelimb movement. In order to put our focus on DHA promoting collateral CST sprouting, unilateral pyramidotomy was employed to address this issue. The pyramidotomy lesion model in mice together with behavioural

tests is a valuable tool for assessing therapeutic strategies to promote CST plasticity (Starkey et al. 2005).

In the unilateral pyramidotomy model, the behavioural data showed that mice treated with DHA made significantly fewer forepaw misplacement 3 weeks after pyramidal tract lesion. The analysis of sprouting CST fibers from the contralateral side shows a robust increase after DHA treatment. Furthermore, the extent of the pyramidotomy lesion was determined using immunostaining for PKCγ in cervical spinal cord. The lack of effect of DHA on PKCγ immunostaining in the CST indicates that there was no neuroprotection of CST axons. Therefore the prominent effect of DHA on the sprouting response of CST axons after pyramidotomy suggests that DHA can promote skilled movement recovery, without a parallel neuroprotection effect. These data provide promising support for neuroplasticity playing a key role in the therapeutic effect of DHA.

However, not all anatomical plasticity is beneficial. From previous investigation, it is known that aberrant neuronal circuits can activate inappropriate combinations of sensorimotor networks during gait execution (Beauparlant et al. 2013). Functional recovery should be built on the basis of direct compensatory plasticity. Previous studies demonstrated that sprouting can result in indirect reconnection of the lesioned axons to caudal targets via propriospinal interneurons (Bareyre et al. 2004; Vavrek et al. 2006). The spinal cord contains many types of interneurons that can be assigned into different categories depending on anatomical, physiological and molecular criteria (Flynn et al. 2011). The C3-C4 propriospinal system has been shown to comprise a population of propriospinal neurons located in upper cervical segments. Its role is to transmit CST input, as well as convergent input from other descending pathways (rubro-, tecto-, and

reticulo-spinal tracts) to motorneurons in segments C6 to T1 that innervate the forelimb (Illert et al. 1981; Alstermark et al. 1984).

In mice, propriospinal neurons include a genetically accessible subpopulation of cervical V2a interneurons (Lee et al. 2001; Al-Mosawie et al. 2007). A recent study has demonstrated that this type of interneuron located in cervical spinal cord is responsible for skilled forelimb movements (Azim et al. 2014). In our study, we utilized the Chx10 transcription factor to localize V2a interneurons. Contact between CST axons and V2a interneurons was confirmed using the confocal microscope. This analysis revealed that DHA can promote sprouting CST axons to contact V2a interneurons. Our work further shows that during CST remodelling not only the number of CST collaterals but also the number of targeted interneurons contacted by CST axons is affected, which is positively associated with functional recovery.

5.5 Summary

- We demonstrate a neuroplasticity promoting effect of DHA in two different animal models.
- DHA can enhance the number of synaptic contacts on motor neurons, which may contribute to functional recovery.
- Two different types of axons (serotonin axons and CST axons) were significantly increased in the spinal cord rostral and caudal to the lesion site after DHA treatment.

- In a mouse pyramidotomy model, DHA treatment promoted skilled locomotor functional recovery and immunochemical staining revealed that sprouting CST fibres contact Chx10 positive interneurons, which correlated positively with functional recovery.

6 The role of PTEN in neuroplasticity modulation after DHA treatment

6.1 Introduction

Utilizing two different CNS injury animal models, we have demonstrated that acute DHA administered as an intravenous bolus 30 min after injury promotes neuroplasticity (chapter 5). While this effect of acute intravenous injection of DHA was supported by data in the previous chapters, there is a need to characterize the underlying mechanisms responsible for neuroplasticity following DHA treatment in SCI. Studies aimed at understanding basic mechanisms of neuronal growth and plasticity are vital in order to develop successful treatments for SCI. Therefore, in this chapter we decided to explore the underlying mechanism involved in DHA promoting anatomical neuroplasticity. In my previous experimental data, delayed (3 weeks post injury) treatment of DHA seemed to have no neuroplasticity promoting effect. The neuroplasticity promoting effect of DHA may thus be only evident at the acute stage.

Several studies have demonstrated that DHA has the ability to modulate gene expression *in vivo* and *in vitro* (Kitajka et al. 2002; Vedin et al. 2012). Owing to the limited time duration of DHA administration after SCI to promote neuroplasticity, it is reasonable to propose that DHA may exert the capacity to modulate gene expression in the early few hours following injury. In several studies, gene expression changes after SCI were analysed using DNA microarrays within a few hours after injury (Carmel et al.

2001; Bareyre et al. 2002; Nesic et al. 2002; Di Giovanni et al. 2003). A very high expression at the epicenter occurred for genes involved in cell damage and death, such as NF-κB, c-jun and suppressor of cytokine signaling 3 (SOCS-3) (Carmel et al. 2001; Song et al. 2001; Di Giovanni et al. 2003). Significantly, transcriptional upregulation of inflammation-related markers was also identified at early time points. The level of pro-inflammatory cytokines (IL-1β and IL-6) and interleukin receptors was elevated threefold (Carmel et al. 2001; Nesic et al. 2002; Di Giovanni et al. 2003). Another group of genes comprised ion channels (e.g. K^+, Na^+, and Ca^{2+} channels) and transporters, which are downregulated one hour after the injury (Carmel et al. 2001; Song et al. 2001; Di Giovanni et al. 2003). Injury of the spinal cord also induces the expression of neuroplasticity genes, such as Janus-activated kinase (JAK), signal transducer and activator of transcription (STAT) family, and insulin-like growth factor (IGF-1) one hour after injury (Nesic et al. 2002). These finding represent an early attempt of the spinal cord towards repair and regeneration.

Currently, there is a lack of direct evidence about gene expression modulated after DHA treatment in SCI models. However, alteration of gene expression following SCI is likely accompanied by the post-transcriptional regulation of these modified gene networks (Nieto-Diaz et al. 2014). miRNAs have recently attracted much attention because of their ability to inhibit mRNA translation, which plays a vital part in post-transcriptional regulation (Pillai 2005). miR-21 has been documented to be an essential factor in multiple biological and pathological processes, including cell proliferation, anti-apoptosis, and inflammation (Krichevsky et al. 2009). The role of miR-21 in CNS injury is not well

documented. A number of studies have identified an increase in the expression level of miR-21 in the hippocampus and injured cerebral cortex from 6 h to 72 h following TBI in rats (Lei et al. 2009; Redell et al. 2009; Redell et al. 2011). The expression of miR-21 is also significantly upregulated following traumatic SCI (Hu et al. 2013; Nieto-Diaz et al. 2014).

Phosphatase and tensin homolog (PTEN), an important target of miR-21, could change the serine/threonine protein kinase Akt phosphorylation status and regulate downstream Akt signalling pathway (Chang et al. 2007). Recently, several studies have demonstrated that downregulation of PTEN function or expression promotes axon regeneration and neuroprotection following CNS trauma (Zhang et al. 2007; Park et al. 2008; Liu et al. 2010; Walker et al. 2012). PTEN is a phosphatidylinositol (3,4,5)-trisphosphate (PIP3) 3-phosphatase, which means it reverses the action of phosphatidylinositol 3-kinase (PI3K) by dephosphorylating PIP3 to PI-4,5-P2 (Cantley et al. 1999). By countering the actions of PI3K, it reduces activation of Akt and prevents all of the downstream signalling events that are controlled by Akt. Inactivation of PTEN leads to accumulation of PIP3 and the activation of Akt.

Previous data have shown that DHA can activate Akt and protect neuronal cells from apoptosis (Akbar et al. 2002; Akbar et al. 2005; Wu et al. 2008; Figueroa et al. 2012). However, the underlying mechanism that activates the Akt signalling pathway still needs further delineation. Work from other labs has shown that PTEN protein expression peaks 24 h post-injury, and the level decreases between 24 and 48 hours following trauma (Ding et al. 2013; Hu et al. 2013). In this study, we tried to determinate if acute administration of DHA can upregulate miR-21 and decrease PTEN expression one day

following cervical spinal injury, and whether this correlates with the promotion of axonal sprouting.

6.2 Aims

In this chapter, our hypothesis is that DHA can initiate a neuroplasticity-promoting effect at a very early stage through miR-21 and PTEN pathways. The aims of this study were to determine whether DHA can induce miR-21 and suppress PTEN expression following cervical hemisection, and to gain more insight into the mechanism of DHA-induced promotion of plasticity (Fig 6.1). In an *in vivo* study, we examined PTEN and miR-21 expression in pyramidal neurons located in layer V of the rat cortex one day following cervical hemisection. In an *in vitro* study, using a PTEN activator (sodium selenite), we have addressed the following questions: first, we tested whether DHA can promote neurite outgrowth in DRG cell culture; second, we examined if DHA could suppress PTEN expression activated by sodium selenite in cell culture and hence promote neurite outgrowth.

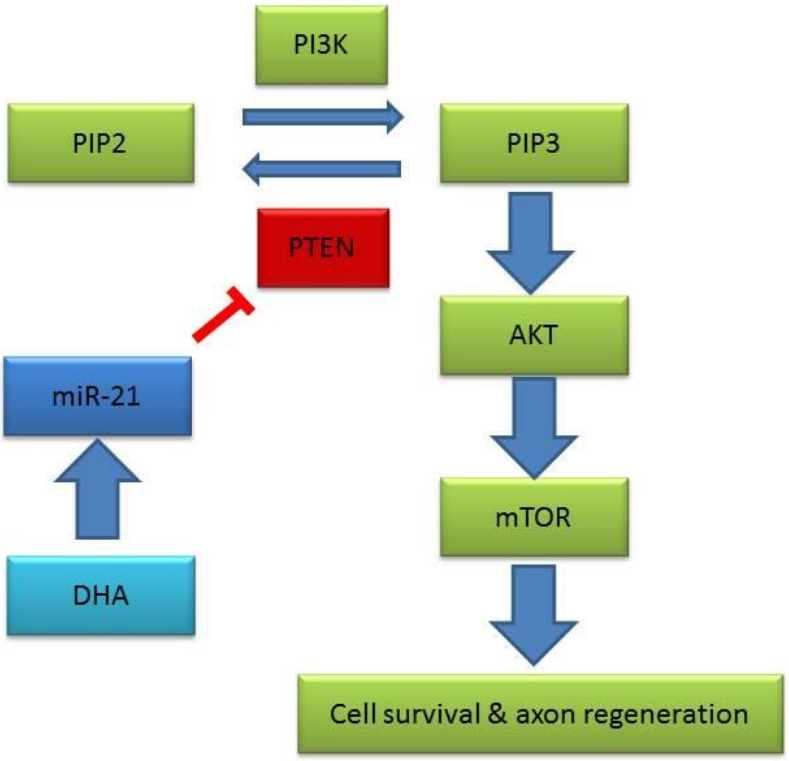

Figure 6-1 Schematic of the hypothesis that DHA upregulates miR-21 which affects the PTEN/mTOR signalling pathway

DHA upregulates miR-21, which inhibits PTEN and therefore activates PI3K/Akt signalling and the mTOR pathway. This process has benefits on cell survival and regeneration. PI3K = Phosphatidylinositol 3-kinase; PTEN = Phosphatase and tensin homolog; mTOR = mammalian target of rapamycin; (adapted from Liu, Detloff, et al 2012)

6.3 Results

6.3.1 Cervical spinal cord injury increases the expression of miR-21

To elucidate the effect of cervical SCI and DHA treatment on miR-21 and PTEN expression in pyramidal neurons in cerebral cortex in the acute stage, cervical hemisection was performed in rats. The rats were separated into two groups. Thirty minutes after surgery, one group received DHA administration and the other group received saline injection. One day after left cervical hemisection, the rats were perfused, and tissue was collected for histological analysis. The CST tract originates from pyramidal cells in layer V of the cerebral cortex. The expression of miR-21 in cortical neurons was measured by *in situ* hybridization. Compared to the left cortex, the miR-21 expression of pyramidal cells was upregulated in the right side cortex after left cervical hemisection in both groups. It is notable that there was a significant upregulation in the vehicle group (Fig 6.1).

6.3.2 DHA induces miR-21 expression in pyramidal cells

To investigate if DHA can induce miR-21, we measured the expression of miR-21 in pyramidal cells of the unlesioned cortex, which is related to CST sprouting. In coronal sections, we discovered that the expression level of miR-21 in pyramidal neurons was significantly different between the two groups. At 1 day after SCI and DHA treatment, the miR-21 expression was substantially increased (Fig 6.2).

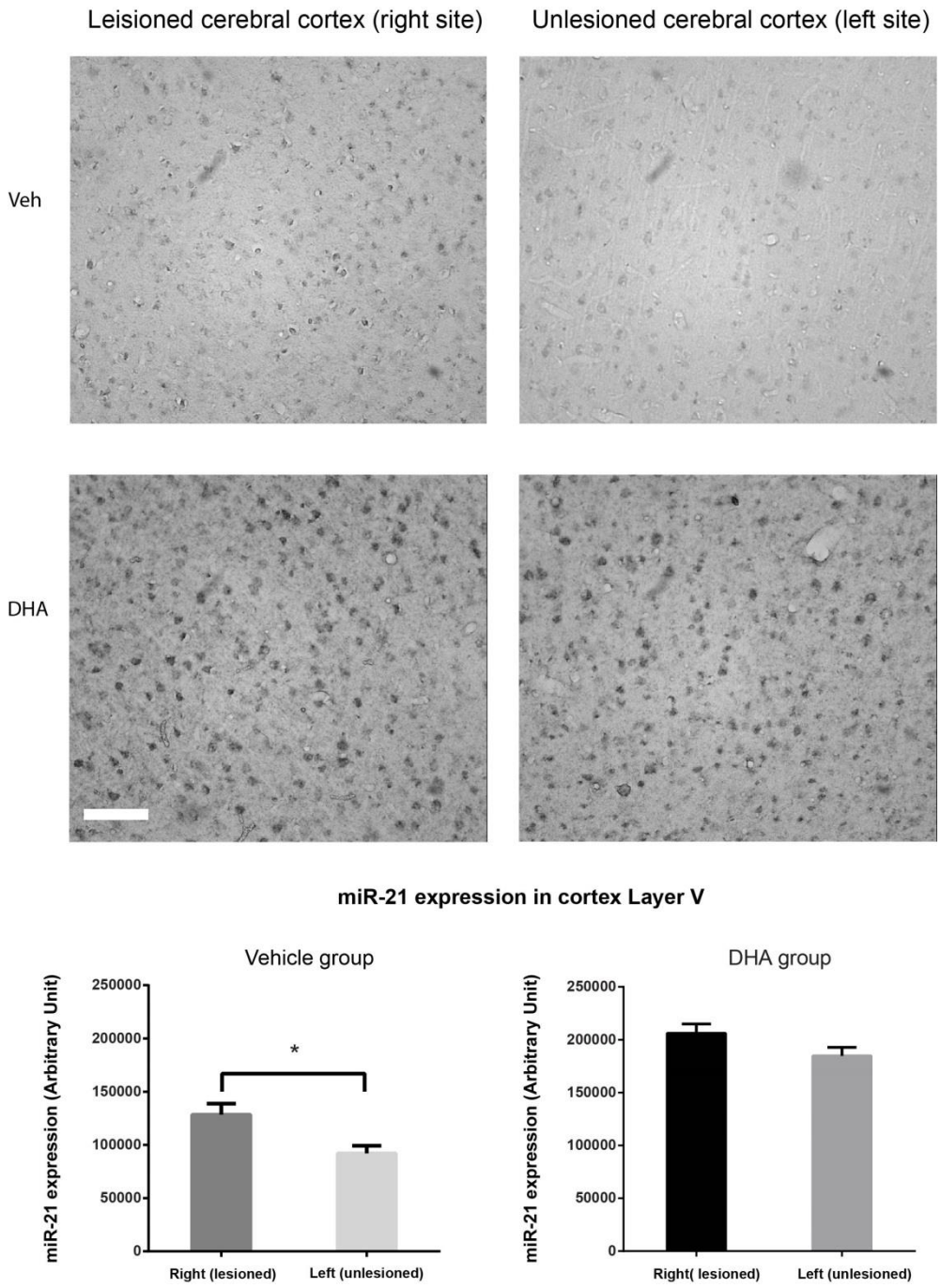

Figure 6-2 Cervical hemisection increases miR-21 expression in cortical neurons

The representative images show miR-21 staining in pyramidal cells in layer V in the cortex, in the vehicle group (A,B) and in the DHA treatment group (C,D). Compared to the left brain cortex, the quantitative analysis revealed that the expression of miR-21 in the right cerebral cortex was upregulated after left cervical hemisection in the vehicle group (C) and in the DHA group (D). Scale bar = 100 μm. A significant difference was noted between each hemisphere in the vehicle group. * $P<0.05$. Results represent mean ±SEM. n=4 animals per group.

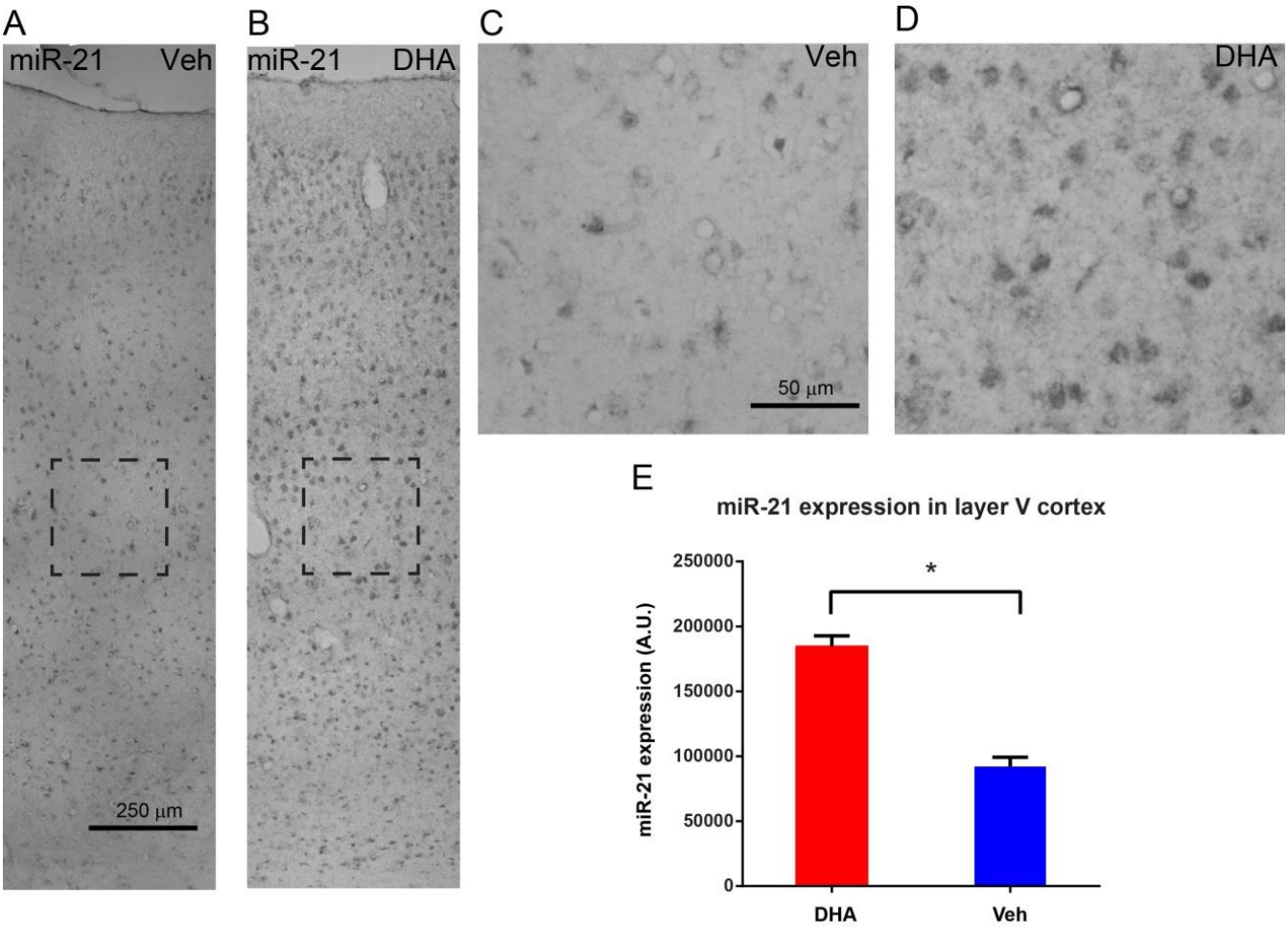

Figure 6-3 DHA treatment increases the expression of miR-21 one day after cervical hemisection

(A, B) Low magnification of the unlesioned cerebral cortex labelled for miR-21 expression in left cervical hemisected rats at 1 day post injury. **(C,D)** Higher magnification of the dashed boxes in panels. **(E)** Quantification of miR-21 expression revealed a significant increase after DHA treatment (* $P<0.05$). Results represent mean ±SEM. n=4 animals per group

6.3.3 DHA treatment decreases PTEN expression in pyramidal cells in motor cortex

In order to assess the effects of the acute bolus DHA treatment on uninjured layer V projection neurons, the PTEN expression in pyramidal cells in motor cortex at bregma +0.14 mm was analyzed one day after cervical SCI. We found that PTEN expression in pyramidal cells was significantly upregulated after cervical hemisection in vehicle group compared to naïve animals. However, the expression of PTEN significantly decreased after DHA treatment compared to the vehicle group (56.5 ± 4.9 vs. 37.8 ± 2.8, $p<0.05$ Fig 6.3).

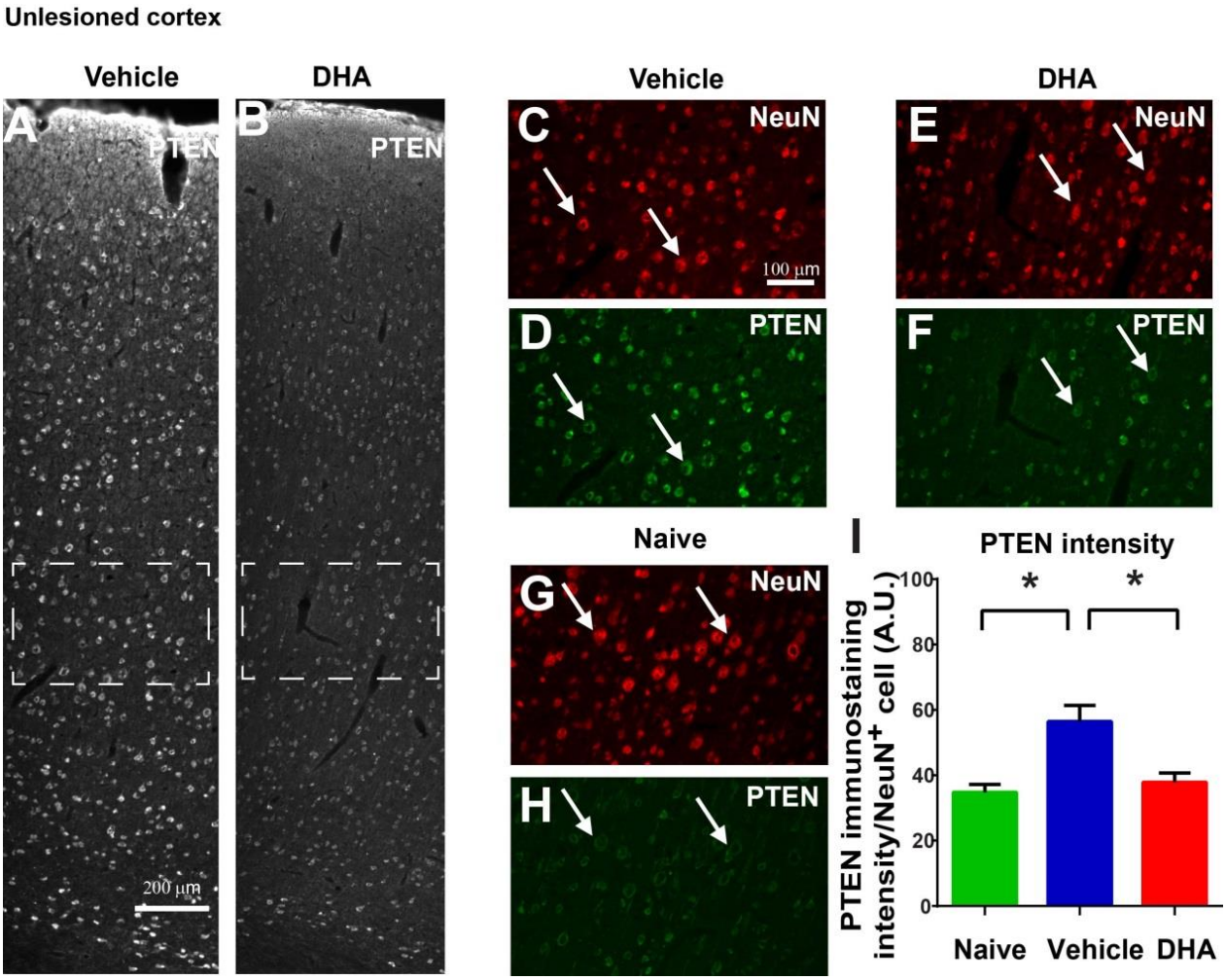

Figure 6-4 DHA suppresses PTEN expression in pyramidal cells

(A,B) Low magnification images of the cerebral cortex labelled for PTEN immunoreactivity in cervical lateral hemisected rats at 1 day post injury. **(C-F)** Higher magnification of the dashed boxes in panels A and B showing that PTEN immunoreactivity is co-localised with NeuN immunopositive neurons in the cerebral cortex. DHA-treated animals have reduced PTEN immunostaining compared to vehicle treated animals. **(G,H)** Low expression of PTEN immunoreactivity was observed in naïve animals. **(I)** Quantitative analysis revealed a significant increase in PTEN immunostaining in the cerebral cortex ipsilateral to the lesion side at one day after cervical SCI in vehicle group.* $P<0.05$. Results represent mean ±SEM. n=4 animals per group. DHA treatment (red bar) significantly reduces the PTEN immunostaining levels when compared to the vehicle treatment (blue bar).

6.3.4 DHA treatment decrease PTEN expression of raphe nucleus neurons in the brainstem

The origin of serotonin projections is in the raphe nuclei in the brainstem. One group of cells (nucleus raphe magnus) is responsible for major spinal cord serotonin projections. In a previous chapter, our data showed the DHA treatment can boost serotonin fiber sprouting following SCI. Therefore, we also determined if DHA can suppress PTEN expression in nucleus raphe magnus, which may promote serotonin fibre sprouting. The response of nucleus raphe magnus following SCI was assessed by NeuN and PTEN immunolabelling of the brain stem.(-8.0 mm vs. bregma) (Abrams et al. 2004) (Fig 6.4). One day after cervical spinal cord hemisection, the PTEN immunoreactivity was decreased in the DHA treatment group in comparison with the vehicle group (Fig 6.5).

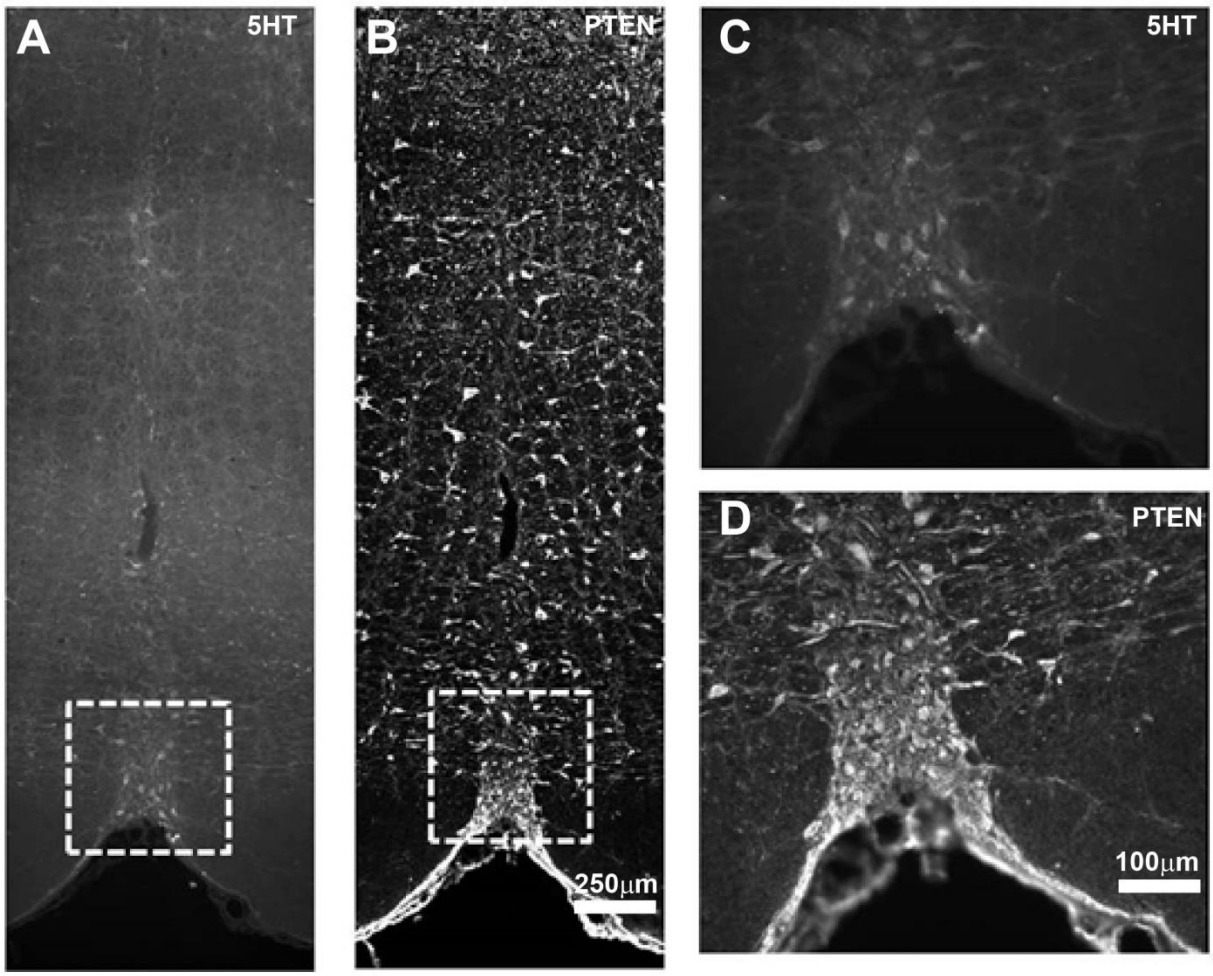

Figure 6-5 PTEN and 5-HT immunoreactivity in the brain stem raphe nuclei

(A,B) Low magnification of the raphe nuclei immunostained for PTEN and 5-HT in cervical hemisected rats at 1 day post injury. **(C,D)** Higher magnification of the dashed boxes in panels A & B, showing PTEN and 5-HT immunoreactivity.

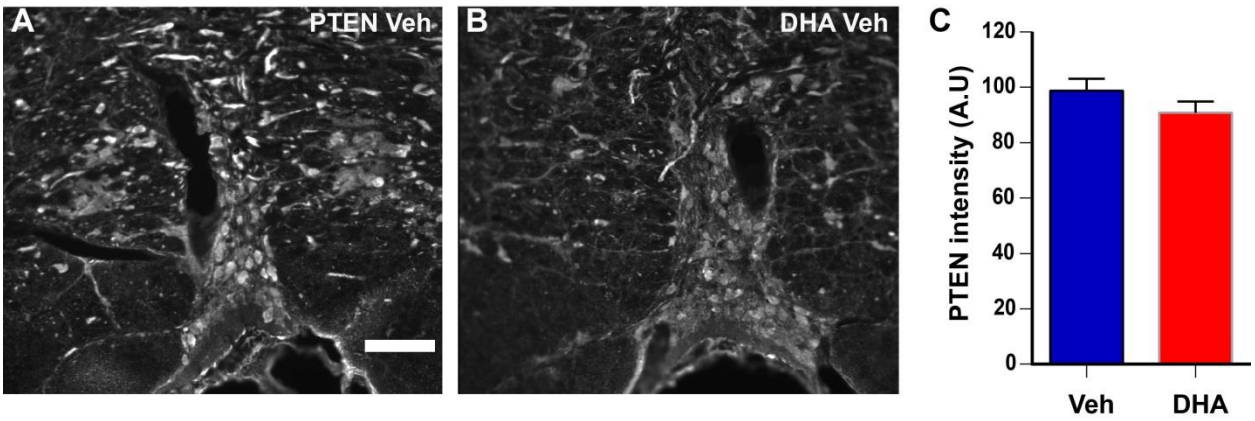

Figure 6-6 DHA treatment induces a small decrease in PTEN expression in nucleus raphe magnus

(A,B) Images of PTEN immunostaining in nucleus raphe magnus. Scale bar =100 μm. **(C)** The quantification showed that DHA induces a trend towards a decrease in PTEN immunoreactivity compared to vehicle group. Results represent mean ±SEM. n=4 animals per group.

6.3.5 DHA enhances neurite outgrowth in DRG cell culture

To evaluate the effect of DHA on neurite sprouting, we examined the growth of DRG cell cultures 3 days following DHA treatment. We compared the effect of different concentrations of DHA on neurite outgrowth. The number of neurites and the average neurite length were significantly increased by 3 μM DHA compared to the control group (Fig 6.7). DHA also increased the maximum neurite length at all the concentrations tested.

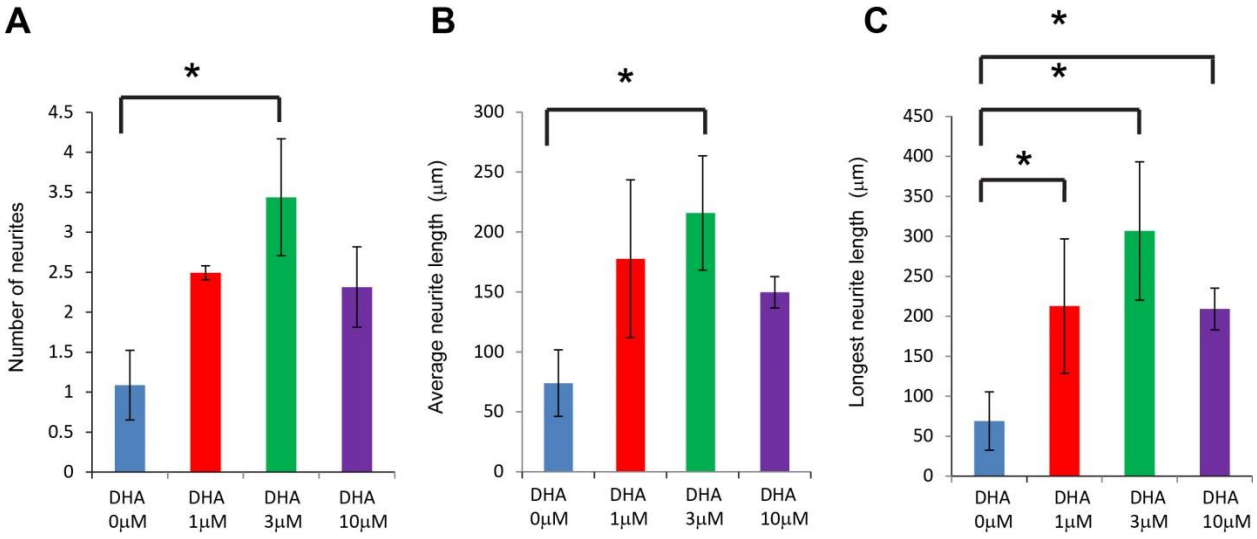

Figure 6-7 DHA promotes DRG cell neurite outgrowth

(A,B) Quantification showed that DHA treatment significantly increases the number of neurites and average neurite length at a dose of 3 μM DHA. **(C)** All concentrations of DHA tested increase the longest neurite length.* P<0.05. Results represent mean ±SEM n=3 independent experiments.

6.3.6 DHA treatment decreases PTEN expression in DRG cell culture.

DRG neurons were cultured as previously described. The next day (1 day *in vitro* (DIV)), DHA (1-10 μM) or control media (BS media with 0.008% ethanol) were added to the DRG cultures. At 2 DIV, sodium selenite (10 μM) was added to the cultures to induce PTEN expression. At 3 DIV, the DRG were fixed with 4% paraformaldehyde and underwent immunocytochemistry. Figure 6.7 shows the induction of PTEN following treatment with sodium selenite. DHA (1 μM) appeared to reduce PTEN expression. This was confirmed by the quantitative analysis, which showed that DHA reduced PTEN expression, in parallel with its neurite growth promoting effect (Fig 6.7).

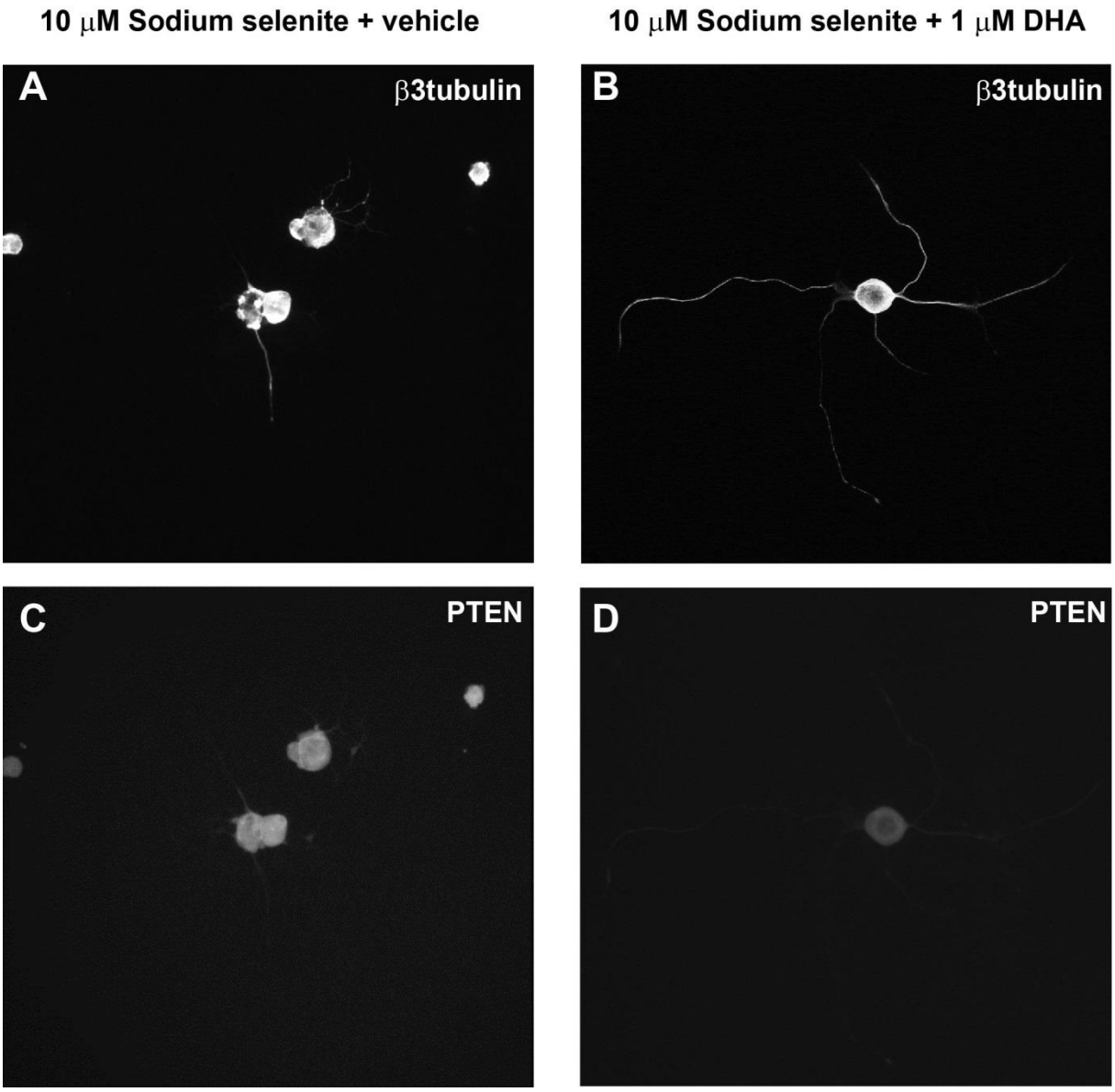

Figure 6-8 DHA treatment reduces PTEN expression and enhances neurite growth

(A,B) In adult DRG neuronal cultures treated with 10 μM sodium selenite, 1 μM DHA promotes neurite outgrowth. **(C,D)** There appeared to be reduced PTEN expression after 1 μM DHA treatment, which occurred in parallel with the effect on neurite growth.

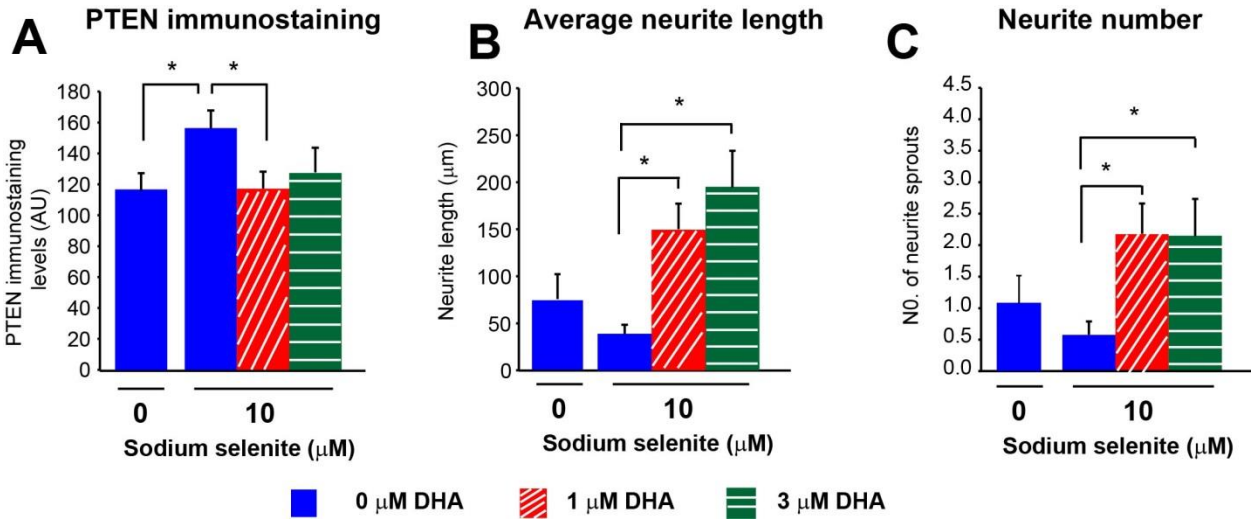

Figure 6-9 DHA treatment reduces PTEN expression and enhances neurite growth

(A) Quantification showed that sodium selenite significantly increased PTEN expression compared to control. DHA significantly decreased the PTEN immunoreactivity at 1 μM. **(B,C)** DHA promotes neurite outgrowth, assessed by average neurite length and neurite number at 10 μM sodium selenite. * $P<0.05$. Results represent mean ±SEM. n=4 independent experiments.

6.4 Discussion

6.4.1 Mechanisms underlying the effect of DHA on neuroplasticity

There are likely to be several mechanisms underlying the effects of DHA on neuroplasticity. DHA has widespread effects on synaptic function. The exact details remain unknown, but are likely to involve complex interactions of synergistic effects on neuronal membrane structure, function, and gene expression. In studies on rodent brain, DHA supplementation increased CaMKII and CREB levels and BDNF secretion to strengthen synaptic plasticity for spatial learning memory formation (Tanabe et al. 2004; Cao et al. 2009). The BDNF system seems crucial for mediating the action of DHA in the brain, as a diet deficient in DHA has been shown to reduce the activation of TrkB receptors (Bhatia et al. 2011). Studies show that the receptor GPR40 is activated by PUFAs (Briscoe et al. 2003; Ma et al. 2007), and a role for GPR40 signalling pathways in adult neurogenesis and the effect of DHA has been proposed (Yamashima 2008). Recent studies have shown that DHA can activate syntaxin 3. Syntaxin 3 is positioned in the presynaptic plasma membrane to detect local changes in PUFA (Darios et al. 2006) and plays a crucial role in the docking and fusion of vesicles during synaptic transmission (McMahon et al. 1995). It also promotes neurite outgrowth by membrane expansion at growth cones (Darios et al. 2006). In one study, DHA treatment also led to an increase in the level of GAP-43 in cortical neuronal culture (Cao et al. 2005). GAP-43 is a protein associated with growth cone formation, which can be used as a marker of axonal growth.

However, the mechanisms mentioned above cannot fully explain my previous data on DHA delayed treatment data or some observations in our group's previous study

(Huang et al. 2007). Thus, the promising functional recovery promoted by DHA was diminished when the single bolus DHA treatment was delayed for 3 weeks or even 3 hours after injury. Therefore, some mechanism promoting axonal sprouting and modulated by DHA is involved only at a very early stage after SCI.

6.4.2 DHA induces miR-21 expression following cervical SCI

MicroRNAs (miRNAs) are highly expressed in mammalian CNS, including in the spinal cord (Krichevsky 2007; Bak et al. 2008) and are integral to many biological processes. miRNAs inhibit translation of mRNAs by leading an inhibitory protein complex, the RNA-induced silencing complex, to the mRNA via complementarity to its 3'- untranslated region (3'UTR). This limits the gene function, even though transcription of the gene may not be stopped (Pillai 2005). Studies published recently have shown that miR-21 is globally upregulated in response to brain injury (Lei et al. 2009; Buller et al. 2010) ,SCI (Strickland et al. 2011; Bhalala et al. 2012; Liu et al. 2012; Hu et al. 2013) and peripheral nerve injury (Strickland et al. 2011; Yu et al. 2011; Sakai et al. 2013). The data on miR-21 suggests that the upregulation of miR-21 attenuates neuronal apoptosis (Buller et al. 2010; Ge et al. 2014) and promotes neuroplasticity after injury, by different signalling pathways (Strickland et al. 2011).

In our in situ hybridization analysis, the expression of miR-21 in the cortex contralateral to the lesion side was augmented compared to the ipsilateral side, after cervical hemisection. This significant upregulation was possibly linked to the degeneration of CST neurons following cervical lesions (Hains et al. 2003; Ghosh et al. 2012) or an attempt by the lesioned cell to regenerate. Furthermore, the rats treated with DHA

showed a higher labelling of miR-21 in cortical pyramidal cells. It seems that the expression of miR-21 was boosted bilaterally in the cortex by DHA treatment.

6.4.3 DHA suppress PTEN expression: *in vivo* study

The manipulation of the level of miR21 has recently been shown to affect functional recovery in animal models of CNS injury by modulation of PTEN expression. In a contusion SCI animal model, knockdown of miR-21 by antagomir-21 led to increased PTEN expression and attenuated neurological functional recovery (Hu et al. 2013). On the other hand, intraventricular infusion of agomir21 in rats receiving traumatic brain injury conferred a better neurological outcome, by inhibition of the expression of PTEN and Akt signalling activation (Ge et al. 2014). These findings suggest that upregulation of miR-21 after DHA treatment may lead to PTEN suppression.

A number of studies have demonstrated that a reduction in phosphatase activity and PTEN is involved in axonal regeneration and synaptic plasticity (Liu et al. 2010; Ding et al. 2013). PTEN is expressed in adult CNS neurons (Cai et al. 2009; Liu et al. 2010) and is essential for processes related to cellular proliferation and neuronal growth regulation (Dahia 2000; Kwon et al. 2001).

PTEN is a PIP_3 3-phosphatase, which means it reverses the action of PI3K by dephosphorylating PIP_3 to PI-4,5-P_2 (Cantley et al. 1999). By countering the actions of PI3K, it reduces activation of Akt and prevents the downstream signalling events that are controlled by Akt. As a result, inactivation of PTEN leads to accumulation of PIP3 and the activation of Akt. The PI3K/Akt signalling pathway regulates crucial biological

processes, including proliferation, growth, migration, metabolism, and neuronal and synaptic plasticity.

PTEN is an early expressed molecule which peaks at 1 day and decreases at 3 days post injury in various rat CNS injury models (Ding et al. 2013; Hu et al. 2013). PTEN over-expression is a possible mechanism to inhibit neuronal regrowth after CNS injury. In our study, the data suggested that DHA can suppress PTEN expression, with a significant decrease in pyramidal neurons in the primary motor cortex region. This finding supports our data showing that DHA can facilitate CST sprouting after cervical hemisection. However, we only found a trend towards decrease in nucleus raphe magnus in the brain stem one day after injury, even though DHA boosts the sprouting of serotonin fibres in the cervical spinal cord following cervical hemisection. A possible reason is that the raphe nuclei are distributed near the midline of the brainstem, along its entire rostro-caudal extent (Hornung 2003). The serotonin projection to the spinal cord mainly originates in the raphe magnus nucleus and terminates in the dorsal horn. PTEN expression in nucleus raphe magnus is easy to identify because of the cell cluster located in the midline. However it is not possible to identify the particular neurons that project to the lesion site. To elucidate this issue, retrograde tracing of serotonin projections fibres may be needed.

6.4.4 DHA suppresses PTEN expression *in vitro*

To investigate the mechanism of PTEN suppression after DHA treatment, we made use of primary cultures of DRG cells. Firstly, we tried to determine the optimal concentration of DHA needed to promote axonal growth. It has been shown that DHA influences the response of neuron growth not only in immature embryonic cell culture (Calderon et al.

2004) but also in mature cell culture (Robson et al. 2010). Our data revealed that DHA can increase the number of neurites and the length of neurites at a concentration of 3 μM DHA. Several mechanisms could underlie the neurite promoting effect. In previous studies, DHA has been shown to upregulate the level of growth associated protein-43 (GAP-43) in cortical neurone cultures (Cao et al. 2005). GAP-43 is a protein associated with growth cone formation. Growth cones are rich in phospholipases which release PUFA from membranes, supporting the importance of these compounds in membrane remodelling and neurite growth (Robson et al. 2010).

In order to investigate if DHA can suppress PTEN expression, we utilized sodium selenite to induce PTEN expression (Berggren et al. 2009; Luo et al. 2013) in DRG cell cultures. The analysis of PTEN immunostaining after sodium selenite incubation revealed a significant increase in the level of PTEN expression in DRG cells and a decrease in the neurite length and number. After incubation with DHA, we saw that the immunoreactivity of PTEN was significantly reduced, and the number and length of neurites was significantly increased by DHA. Our result implies that inhibition of PTEN expression promotes neurite growth in DRG cell culture. This interpretation is consistent with other study results, which demonstrate that inhibition of PTEN in DRG cell culture promotes neurite outgrowth (Christie et al. 2010).

From our *in vivo* and *in vitro* data, we found therefore that PTEN suppression is associated with the elevation of miR-21 after DHA treatment. This data is similar with another study result. In cortical neuron cultures, a study has demonstrated that miR-21 can inhibit the expression of PTEN through Akt/PI3 pathway after scratch injury (Han et

al. 2014). This may be one potential mechanism which explains why acute DHA treatment can promote axonal sprouting after cervical SCI.

6.5 Summary

- In an *in vivo* study, increased miR-21 expression was observed in CST neurons after cervical SCI and DHA treatment.
- Acute administration of DHA inhibits PTEN expression in pyramidal cells and nucleus raphe magnus one day after cervical hemisection.
- In DRG cell culture, DHA increases the number of neurites and the length of neurites.
- In DRG cell cultures, DHA decreases induced PTEN expression and enhances neurite outgrowth
- The *in vivo* and *in vitro* results provide evidence that DHA can upregulate miR-21 and inhibit PTEN expression after cervical hemisection, which may contribute to neuroplasticity.

7 Effect of combined DHA treatment and rehabilitation training in cervical SCI

7.1 Introduction

SCI results from an external force to the spinal cord causing transient or permanent neurological dysfunction of motor, sensory, and autonomic systems at and below the lesion site. Gradual recovery of neurological function does occur in the first year following incomplete SCI (Burns et al. 1997; Scivoletto et al. 2009). Rehabilitation therapies, such as intensive repetitive training (Beekhuizen et al. 2005) and locomotor training (Behrman et al. 2006) have shown great potential to promote functional recovery in patients. Such training appears to amplify the spontaneous axonal sprouting, cortical map reorganization and growth-associated protein up-regulation that develop following SCI.

As the effect of rehabilitation training is modest, it appears desirable to combine pharmacological treatments which promote plasticity with training in order to maximize the plasticity effect. There is a need to develop novel single and/or combined treatments aimed at promoting neuroplasticity events following SCI and thus enhance functional recovery. From the results of chapter 5 and chapter 6, the neuroplasticity promoting effect of DHA appears promising. Moreover, acute DHA treatment with task-specific rehabilitation had not been investigated in a rodent model of cervical SCI. In this chapter, the effect of voluntary task-specific training (Montoya staircase) and of DHA, either alone or in combination, on neurological functional recovery was examined in a rat model of cervical hemisection.

7.1.1 Task-specific training

Recently, several experimental studies have demonstrated that specific task training enhances forepaw reaching and grasping ability following different partial lesions of cervical spinal cord and pyramidal tracts (Girgis et al. 2007; Krajacic et al. 2010; Starkey et al. 2011). Recovery of forepaw function has been linked to plasticity in various descending systems, including the CST and the rubrospinal tract (Krajacic et al. 2010). However, the training benefits vary depending on the lesion type. Rats with a lesion involving the dorsal column that received training, showed significantly greater improvement compared to untrained animals. However, rats with a lateral funiculus lesion did not show any training effect (Krajacic et al. 2010). To our knowledge, there is no literature to discuss the benefit of specific task training in a cervical hemisection animal model. In order to design effective and relevant neuroplasticity promoting strategies, we need to explore the therapeutic effect of training in a range of animal models.

7.1.2 Combined therapy

In the past, different therapeutic strategies have addressed one particular target, such as neuroprotection, neuroregeneration, or neurorehabilitation. However, many recent studies indicate that a greater effect may be achieved when a combination of interventions is implemented. For example, in a thoracic SCI animal model, treadmill training combined with OEG cell transplantation (Kubasak et al. 2008) and radiation therapy (Ichiyama et al. 2009) demonstrated significant improvement compared to

individual therapy. A number of studies also showed that voluntary forepaw motor rehabilitation shows a strong synergistic effect when given together with CSPG digestion (Garcia-Alias et al. 2009; Ichiyama et al. 2011) or neurotrophic factor treatment (Weishaupt et al. 2013) in a cervical SCI model.

Although combined therapy following SCI benefits neurological recovery in most studies, undesired effects of training are also reported in a few studies. Combined with Nogo neutralization in partially lesioned rats, intensive training appears to interfere with functional recovery, suggesting competition between mechanisms when both strategies are applied together (Maier et al. 2009). Another report showed that animals receiving either step training or neurotrophins alone improved to a similar locomotor performance, while the combined therapy did not lead to significant recovery compared to the individual interventions (Boyce et al. 2007). It is postulated that step training exerts much of its effect through neurotrophins, so there is little synergistic effect when the two interventions are combined.

Recently, data from another laboratory revealed that a combination of exercise and dietary DHA promoted a significant enhancement in cognition in naïve animals or after TBI compared to either intervention alone (Wu et al. 2008; Wu et al. 2013). These results appear to be related to a synergistic effect of combined therapy via a BDNF-mediated mechanism and an increase in the level of GAP43 and syntaxin-3 in the hippocampus (Chytrova et al. 2010). It therefore seems logical to examine the synergistic effect of DHA treatment along with a rehabilitation program in our cervical SCI animal model.

7.2 Aim

In previous chapters, we demonstrated that acute DHA administration enhances neuroplasticity in our cervical hemisection animal model. In this chapter, the hypothesis is that combined DHA and rehabilitation treatment can achieve better neuroplasticity-promoting effects. We applied task-specific training in our animal model to examine the rehabilitation effect on neurological functional recovery. In addition, we sought to test whether promoting spinal cord plasticity with combined rehabilitation and DHA therapy maximizes functional recovery.

7.3 Results

7.3.1 Rehabilitation promotes forelimb skilled motor recovery following cervical hemisection

One objective of this chapter was to examine whether task-specific training on the Montoya staircase in rats receiving cervical hemisection could improve functional recovery. Therefore, two groups of rats underwent 3 weeks rehabilitation starting 2 days after cervical hemisection. In one group, rats received DHA 250 nmol/kg 30 min after cervical hemisection, and in the other group rats received a saline injection as in my previous study design. After 3 weeks of behavioural testing, the animals were sacrificed and spinal cord tissue was harvested for further histological examination.

Compared to our previous animal Montoya staircase data, our results show that in rats receiving cervical hemisection with/without DHA treatment, 3 weeks rehabilitative training increased the neurological functional recovery (Fig 7.1). In the saline vehicle group, trained rats significantly grasped more food pellets 10 days after cervical hemisection. A similar trend was seen when the number of food pellets eaten by trained rats with DHA treatment was quantified. Interestingly, the training did not increase the ability of rats receiving DHA treatment to displace more food pellets. It appears that training increases the accuracy and success of food retrieval 12 days following cervical hemisection.

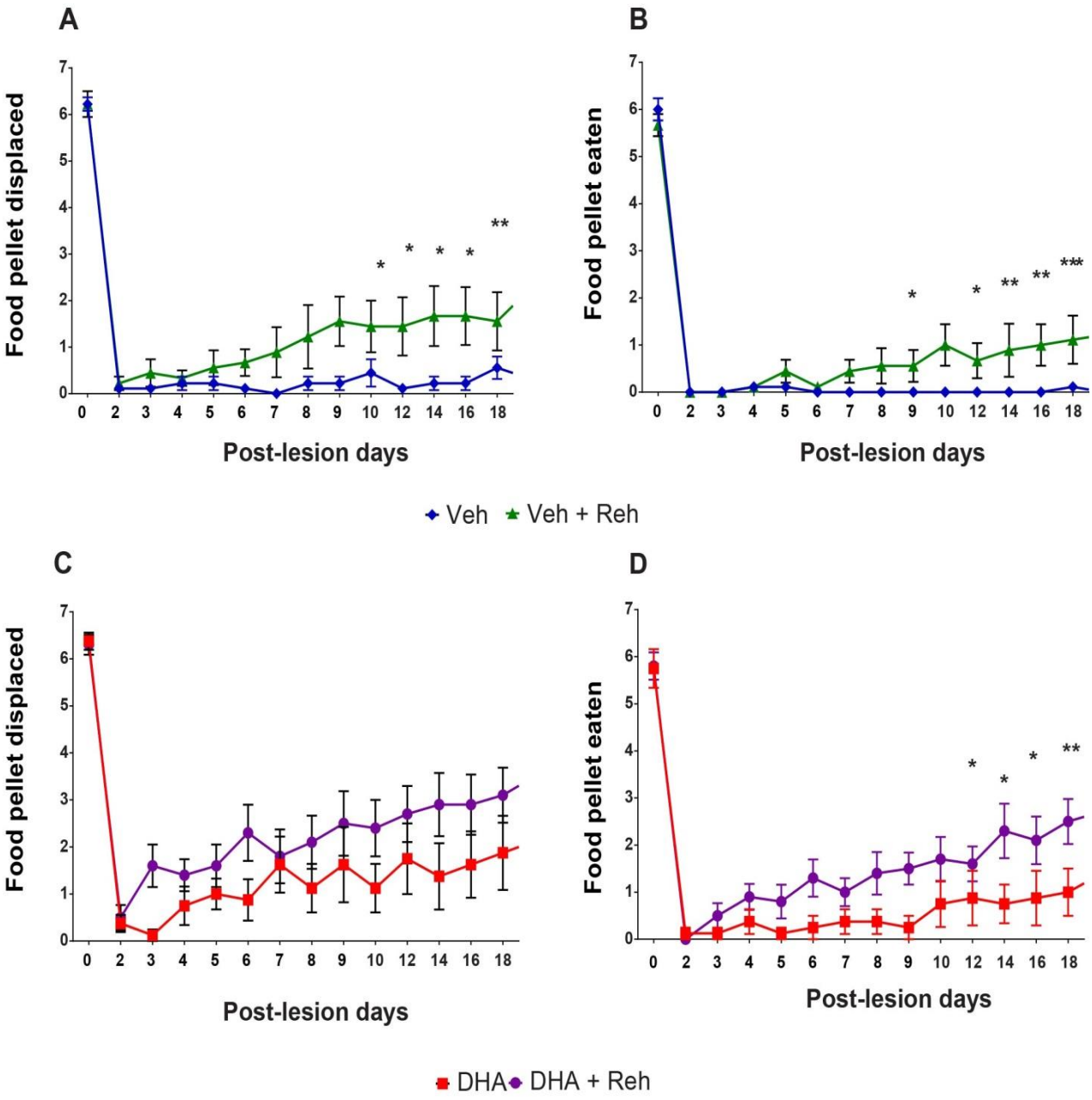

Figure 7-1 Effect of rehabilitation training on skilled forelimb function

After SCI, all rats lost grasping ability in the Montoya staircase test. Starting from 10 days, rats receiving rehabilitation training began to displace and retrieve significantly more pellets in the vehicle group **(A,B)**. In the DHA treatment group, rats receiving rehabilitation training displaced more food pellets compared to untrained rats overall. However, there was no significant difference between these two groups. 12 days after lesion, the rats grasped more food pellets compared to the untrained rats **(C,D)**. (* $P<0.05$,** $p<0.01$, *** $p<0.001$). Result represent mean ± SEM; n=8-10 animals in each group.

7.3.2 Combined therapy induced greater skilled forelimb functional recovery than single treatment

Concerning food pellets eaten, at the time point of 3 weeks after cervical hemisection, those animals receiving combined therapy were performing significantly better than the other three groups, receiving DHA treatment alone, rehabilitation training alone, or the control group (Fig 7.2). This shows that rehabilitation or DHA treatment alone enabled animals to eat more food pellets compared to vehicle group during test, but the combination of DHA and rehabilitation enabled animals to grasp and eat more food pellets than each treatment alone.

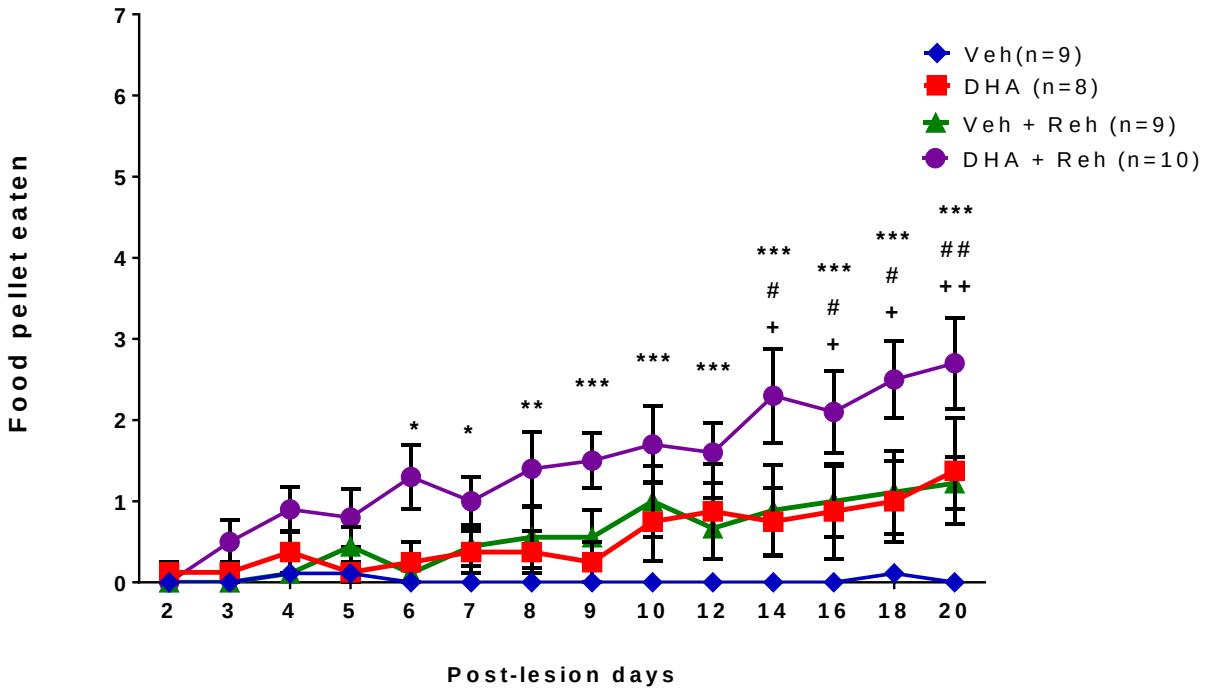

Figure 7-2 Effect of combined therapy on skilled forelimb function

For the first 48 hours after surgery, no rats showed any ability to retrieve food pellets. At 6 days post-injury, DHA-treated rats receiving training had begun to outperform the other groups. Animals receiving DHA treatment with task-specific rehabilitation began a gradual recovery, which started to diverge from the DHA or rehabilitation only groups around 2 weeks after cervical hemisection. (* represents ***DHA + Reh*** vs ***Veh***, **#** represent ***DHA+Reh*** vs ***DHA***, **+** represents ***DHA + Reh vs Veh + Reh,*** * P< 0.05, ** P< 0.01, *** P<0.001, # P<0.05, ## P<0.01, + P<0.05, ++P<0.01) Result represent mean ± SEM; n=8-10 animals in each group.

7.3.3 Rehabilitation training has no effect on skilled locomotion recovery

The grid exploration is also designed to assess fine motor control of the forelimb, including wrist and paw, but it is essential to consider that walking over rungs is still a fundamentally different motor skill than reaching for pellets. Animals were placed on the grid one, two and three weeks post-operatively, to assess the misplaced steps made by the injured left forelimb. After injury, overall the rats receiving rehabilitation training without treatment had fewer misplacements compared to the control group during 3 weeks. However, there was no significant difference between animals receiving rehabilitation training or not. In the DHA treatment groups, no obvious difference was found during this skilled locomotion test, with or without rehabilitation.

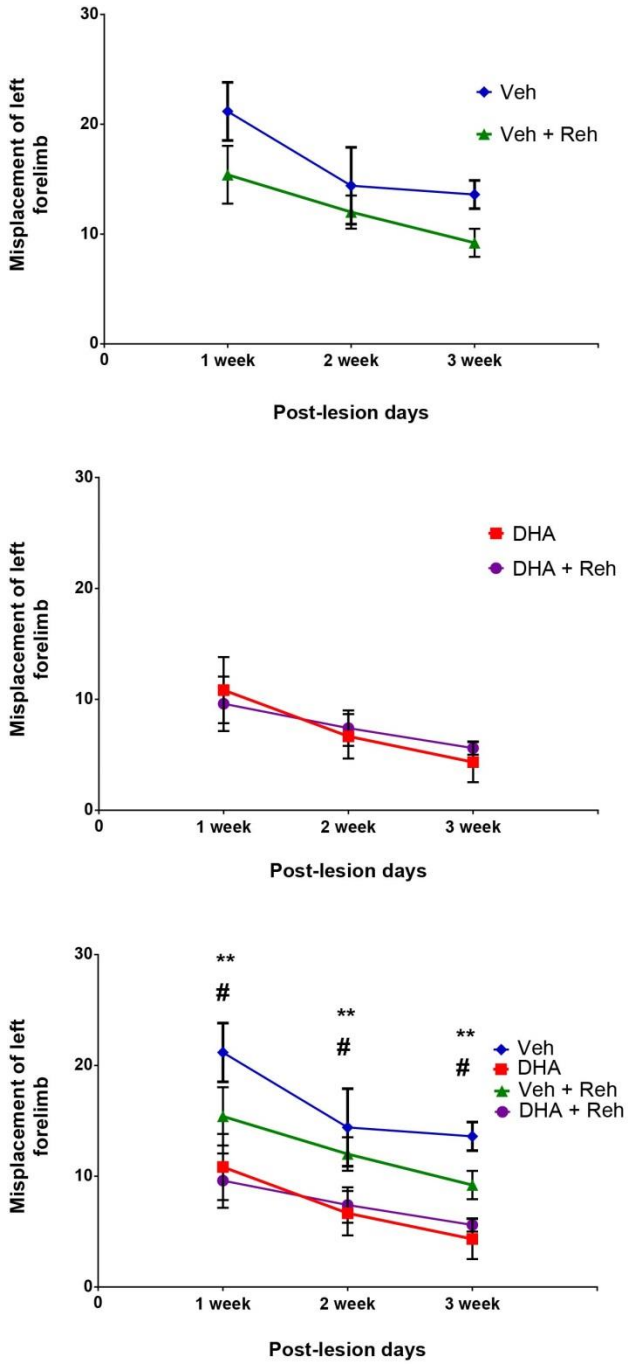

Figure 7-3 Effect of combinatorial treatment on non-trained skilled movement.
In the vehicle group, overall, the rats receiving rehabilitative training exhibited fewer forelimb misplacements during the test period. However, no significant improvement in this test was found vs. the control vehicle. In the DHA treatment groups, the number of forelimb misplacements decreased week by week. The performance in the grid exploration test showed no obvious effect of rehabilitation training in combination with DHA. Overall, the rats receiving DHA treatment made fewer misplacements. Results represent mean ± SEM; n=6 animals in each group. (* represents DHA + Reh vs Veh, # represent DHA + Reh vs Veh +Reh * P< 0.05, ** P< 0.01, # P<0.05)

7.3.4 Combined therapy promotes axonal sprouting

7.3.4.1 Corticospinal axons

The anatomical changes in the contralateral spared CST were studied following ipsilateral BDA injection into the forepaw representation area of the sensorimotor cortex. We quantified contralateral CST sprouting across the midline above and below the lesion site. In the vehicle group, the rehabilitation training promoted significant CST fibre sprouting above and below the lesion site (Fig 7.4). In the DHA treatment group, there was a similar pattern of CST fibre sprouting. Both groups receiving rehabilitation training regardless of DHA treatment showed increased axon sprouting across the midline compared to the vehicle group.

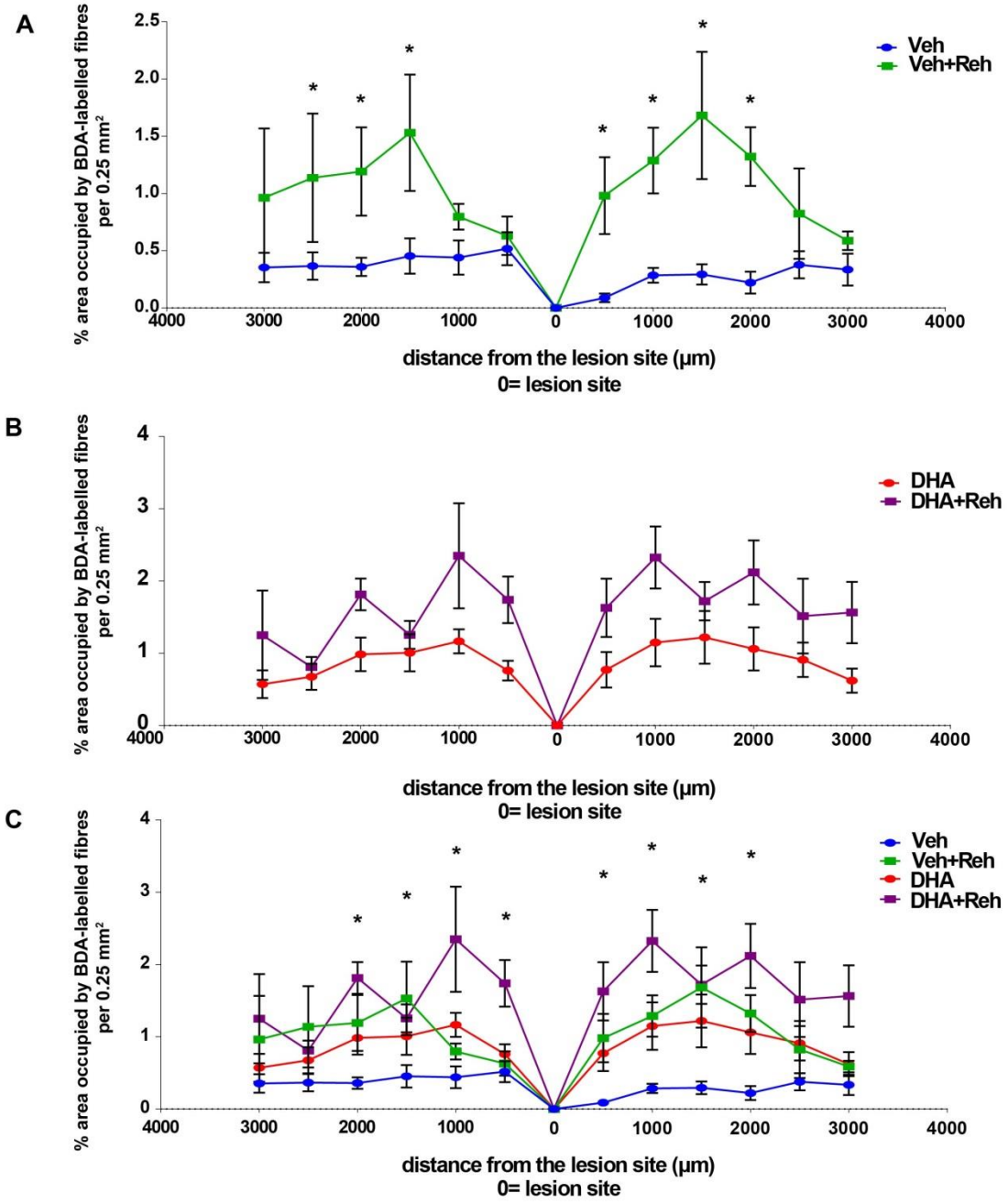

Figure 7-4 The effect of rehabilitation training on CST axon sprouting
The CST sprouting fibres in the spinal cord rostral and caudal to the lesion site were quantified. **(A)** The data revealed large numbers of sprouting axonal fibres in rats receiving rehabilitative training in the vehicle group (* $p<0.05$). **(B)** In the DHA treatment group, overall the training enhanced the sprouting of CST axonal fibres moderately. There was no significant difference in CST sprouting between the two DHA treated groups. **(C)** The data for all the four groups demonstrates a trend toward DHA-treated rats with rehabilitative training having more CST sprouting than the other groups. (*$P<0.05$, DHA + Reh v.s Veh). Results represent mean ± SEM; n=3-6 animals in each group.

7.3.4.2 Combined therapy and serotonergic fibre sprouting

The sprouting of other CNS axons was also examined to reveal whether or not sprouting was restricted to corticospinal fibres. Utilizing 5-HT immunohistochemical staining, serotonergic axons were identified. To investigate the sprouting axon fibres below the lesion, the number of serotonin fibres was quantified 5 mm caudal to the lesion site. The quantification showed that animals that received combined therapy had significantly more axons caudal to the lesion, indicating a greater increase in the sprouting of serotonergic axons after combined treatment (Fig 7.5).

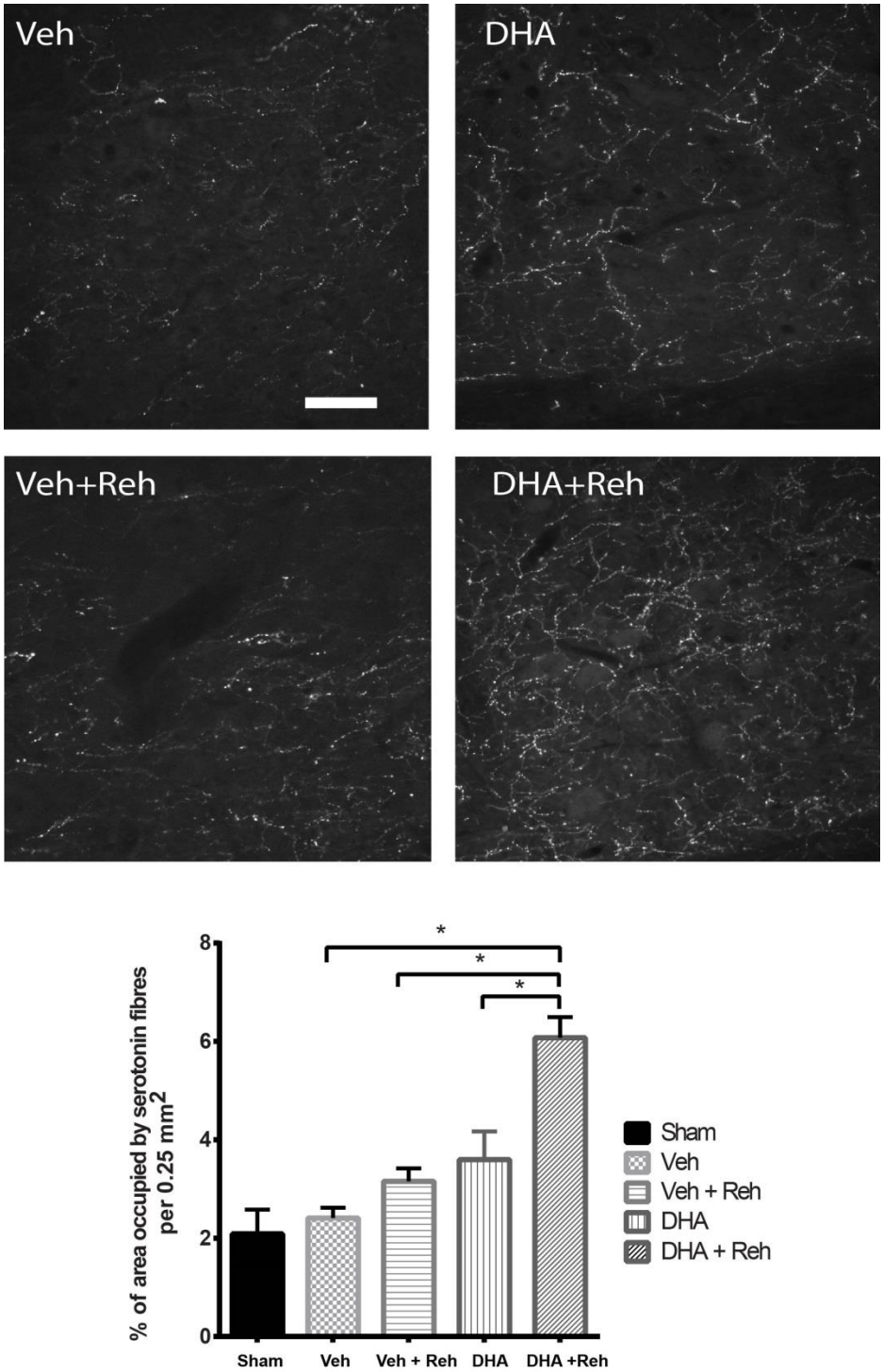

Figure 7-5 Effect of combined therapy on serotonin fibres
Representative images of 5-HT labelling in the dorsal horns 5 mm caudal to the injury site in rats receiving DHA and saline treatment with/without rehabilitation training. Scale bar =100 μm. The quantification showed that combined therapy significantly increased the density of serotonin fibres (* $p<0.05$). Result represent mean ± SEM; n=5 animals in each group.

7.3.5 The effect of combined therapy on synaptogenesis

Synaptic terminals around motor neurons were identified by the level of synaptophysin. Synaptophysin is downregulated after SCI (see chapter 5). Given that DHA promoted synaptic plasticity after SCI, we asked whether combined therapy could also maximize the expression of synaptophysin. As shown in Fig. 7.6, using the immunochemical staining method, we observed the expression of synaptophysin around ventral horn neurons located in spinal cord 5 mm caudal to the lesion site. In saline and DHA-treated groups, the rehabilitation training increased the level of synaptophysin; however there appeared to be no significant synergistic effect. Overall, the combined therapy led to the highest level of synaptophysin surrounding motor neurons (Fig 7.6).

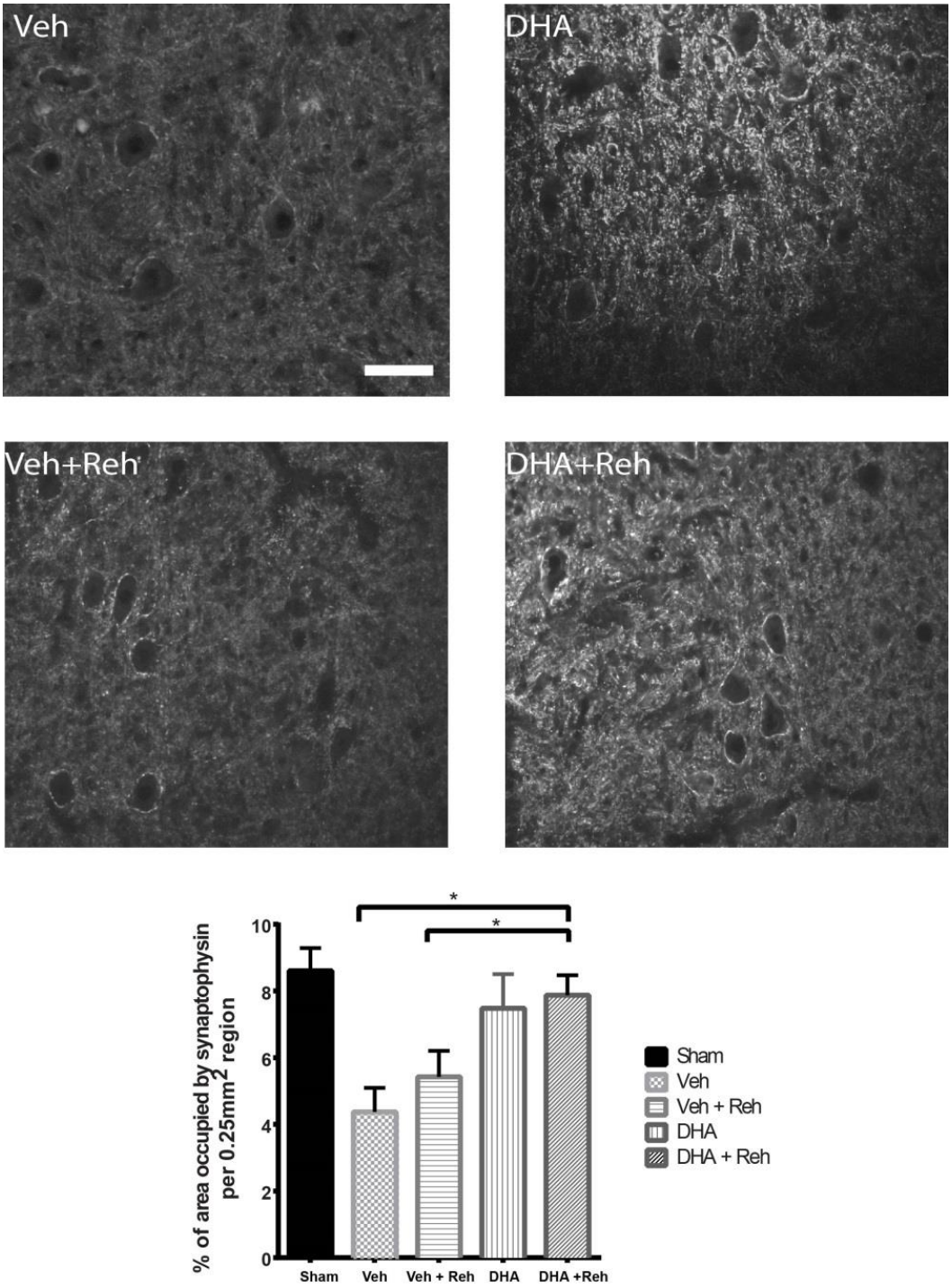

Figure 7-6 Effect of combination treatment on synaptogenesis
Representative images in the cervical ventral horn taken 5 mm caudal to the epicenter, in DHA and vehicle treatment rats, with/without rehabilitative training. Sections were immunostained with synaptophysin at 3 weeks after SCI. Quantitative analysis revealed a significant increase in the DHA treatment group receiving training versus vehicle group with/without training (* $p<0.05$). Result represent mean ± SEM; n=5 animals in each group. Scale bar =100 μm.

7.4 Discussion

The results presented here show that the neuroplasticity following cervical hemisection, in terms of both functional neurological manifestations and histological outcome, is significantly improved after combined therapy compared to single treatment. Specific forelimb task behavioural assessments showed better functional recovery in rats receiving intensive rehabilitation training combined with DHA treatment. However, another skilled behavioural test (grid exploration) did not reveal a significant effect of rehabilitation training. It is clear from our data that the acute administration of DHA combined with early rehabilitation greatly increases synaptic plasticity and nerve sprouting in the spinal cord caudal to the lesion site.

7.4.1 Rehabilitation effects following cervical SCI

In this chapter, we showed that specific-task training in rats following cervical hemisection can significantly enhance the recovery of that particular task regardless of DHA treatment. Rehabilitation training is a non-invasive therapeutic approach with the potential for profound effects in SCI patients. From animal SCI experimental studies, it is clear that training can promote plasticity and functional recovery by inducing cellular and molecular changes.

Probably the most established and investigated rehabilitation paradigm following SCI is treadmill training, which has been successfully translated into a clinical treatment. Training effects have been seen in experimental mice (Goldshmit et al. 2008), rats (Fouad et al. 2000; Fouad et al. 2004), and cats (Rossignol 2006) as well as in humans

(Wernig et al. 1992; Dietz 1995; Dietz et al. 1998). Recently, the promising effects of task-specific training were demonstrated in rodents following cervical SCI with or without pharmacological treatment (Girgis et al. 2007; Krajacic et al. 2010). An essential difference between these two methods is that forelimb task-specific training involves fine motor control that is directly organized by the brain and brainstem centres. On the other hand, repetitive locomotor training is modulated by central pattern generators.

Successful food pellet retrieval is required for animals to pick-up sugar pellets and put them in their mouth. The wells in Montoya staircase are small and deep, and the rats need to use their digits to winkle the pellets out of the wells and pick them up. In the initial stage of forelimb functional recovery, the rats recovered gross forelimb movement but still lacked the ability to grab the food pellets successfully with fine control. The food pellets were flicked out of the wells during the test. In our data, the rehabilitation training significantly increased the number of food pellets displaced and eaten in the Montoya staircase test. Recovery of this substantial skilled task has been correlated to the number of collateral CST sprouts after treatment (Krajacic et al. 2010). With DHA treatment, the displacement of food pellets seems not significantly different between trained and untrained animals. However, the number of food pellets eaten showed a significant increase after 2 weeks rehabilitation training. Such data add to the view that the rehabilitation did not improve gross movement, but led to a significant success in food retrieval, with delicate digit control after DHA treatment. Interestingly, the histological analysis of the number of collateral CST sprouting axons in trained rats' cervical spinal cord did not reveal any significant enhancement. It is postulated that

rehabilitation training strengthens good connections and removes the incorrect connections, to achieve the synergistic effect of combined therapy (Fawcett et al. 2009).

Some studies report that the training-induced improvement in one particular task may be at the cost of an untrained task (Girgis et al. 2007; Garcia-Alias et al. 2009). This supports the hypothesis that there is a limited resource for new nerve circuit formation following SCI, and different behaviours may compete for that resource (Garcia-Alias et al. 2012). In order to address this question, we employed the grid exploration test to examine if task-specific training interfered with another untrained skill. Interestingly, in the vehicle group, we found that rats receiving training made fewer foot slips compared to untrained rats. In the DHA treatment group, there was no significant difference. Our data are similar to the results from other groups. After cervical lesion, rats receiving forelimb skilled grasping training make fewer mistakes while running along a ladder or beam (Krajacic et al. 2010; Wang et al. 2011).

In one study, after unilateral pyramidotomy, rats were trained on either the single pellet grasping or the horizontal ladder task. Single pellet grasping training led to a smaller improvement on the horizontal ladder, but the same degree of recovery on the single pellet grasping task as horizontal ladder trained animals. Anatomically, only single pellet grasping training was associated with enhanced sprouting of the intact corticospinal tract across the midline of the cervical spinal cord to innervate the denervated side (Starkey et al. 2011). These results imply that skilled paw reaching, which relies heavily on the CST, only recovers with treatment promoting CST sprouting. However, the grid

exploration test, ladder walking or other less skilled tasks, may also involve other spinal pathways. Promoting CST sprouting is not sufficient to fully support functional recovery in these tasks.

7.4.2 Histological changes following rehabilitation training

A possible mechanism contributing to functional recovery is the sprouting of lesioned and spared CST fibres. Several studies have revealed that rehabilitative training alone or in combination with other therapies promotes reorganization of the CST after SCI (Fouad et al. 2000; Girgis et al. 2007; Krajacic et al. 2010). In the vehicle group, we found that contralateral sprouting of CST axons was enhanced in animals that underwent rehabilitative training. In the DHA treatment group, the difference in sprouting CST fibres was not obvious between trained and untrained groups. Although our approach of quantifying midline-crossing collaterals in the cervical spinal cord did not provide evidence for the desired rewiring in the combined treatment group, this does not prove that there is no beneficial re-arrangement of the CST. Already established connections might merely be strengthened, or primary collaterals might branch and create secondary collaterals within the vicinity of the lesion site, to increase connectivity.

It is unlikely that sprouting of CST fibres is the only mechanism for the restoration of forelimb function. This enhancement could be achieved by an increase in other axon sprouts and/or by increasing the number of receptors at functional synapses. Thus, we focused on the change in synaptic terminals and in serotonin fibres in the spinal cord caudal to the lesion site, where motor neurons are located related to fine motor control.

The analysis of serotonin fibres 5 mm below the lesion site revealed that training significantly increased serotonin fibre density. This data is consistent with previous studies (Wang et al. 2011). The possible explanation of how combined therapy can increase the serotonin fibres is that brain derived neurotrophic factor (BDNF) is upregulated after DHA and exercise (Wu et al. 2008). BDNF is associated with structural changes including increased sprouting of serotonergic axons in the rat brain (Mamounas et al. 1995). Previous studies demonstrated that BDNF secreting grafts enhance sprouting of serotonergic fibres at the site of injury, and that exercise can induce increased serotonin fibre density in the lumbar spinal cord of mice after moderate contusion injury (Engesser-Cesar et al. 2007).

7.4.3 Timing of training

The current work demonstrates that early task-specific rehabilitation improved functional outcome in rats following cervical hemisection, which correlated with increased axonal sprouts and synaptic terminals. Several studies have documented that rehabilitation is more efficacious when carried out within a few days after injury (Norrie et al. 2005; Winchester et al. 2005). Various potential issues for the decline in responsiveness after delayed rehabilitation training have been addressed. An early approach is considered most effective as injury induced upregulation of various growth-promoting factors is only transient (Hayashi et al. 2000; Song et al. 2001; Di Giovanni et al. 2005). The expression of plasticity promoting genes and growth factors, which are upregulated immediately following SCI (Song et al. 2001; Di Giovanni et al. 2005; Jacobi et al. 2014), declines over time. Another potential reason is the increase in growth inhibitory

proteoglycans after SCI (Massey et al. 2008). It is also notable that degradation of neuronal and muscle function occurs following SCI (Dietz et al. 2004). Axonal injury triggers degeneration of severed axons within 2-3 days (Court et al. 2012). However, a previous study showed that swimming rehabilitation delayed by 2 weeks post-injury is more effective than delivery at 3 days (Smith et al. 2006). In addition, the authors observed increased extravasation at the epicenter of the lesion, suggesting increased secondary damage and neuronal loss in the 3 day group. In further studies, we could explore the temporal aspect of intervention with rehabilitation training in order to maximise our treatment strategy.

7.4.4 Synergistic effect of DHA and rehabilitation

The most compelling effect of treatment on motor function was achieved when DHA was combined with rehabilitative training. How might DHA and rehabilitation work together to promote functional recovery? One reason why training has such an impact on the emergence of DHA effects might be the fact that any plastic changes in the nervous system rely heavily on activity for development, maintenance and fine-tuning of new synaptic connections. This enhancement can be promoted by the increased activity of existing receptors and /or by increasing the number of receptors at functional synapses. A role for DHA in synaptic plasticity is well established in both *in vivo* and *in vitro* studies. To evaluate a possible functional role for synaptic upregulation, we measured the level of a presynaptic protein (synaptophysin) in the cervical spinal cord. The levels of synaptophysin were significantly elevated in the spinal cord of rats receiving combined treatment; however, the elevation was not linked to a significant difference in

functional outcome between the control and rehabilitation only groups. Although the expression of synaptophysin was not significantly elevated in the cervical spinal cord in the rehabilitation group, it would be interesting to explore if rehabilitation can upregulate other synaptic proteins such as bassoon, which is an established active zone marker protein for the synapses of the CNS (Dondzillo et al. 2010; Chen et al. 2012).

As an alternative to the hypothesized synaptic up-regulation, what other mechanisms might mediate the observed significant benefits of DHA treatment on motor recovery? A secondary effect is to promote sprouting of axons above and below the lesion. We observed a similar magnitude of CST sprouting in single treatment and combine therapy. However, the significant sprouting of serotonergic axons was only detected in the combined treatment. A possible mechanism to promote axonal sprouting is BDNF upregulation after combined treatment. There are a few studies indicating that training can upregulate expression of BDNF in the nervous system and that this is pivotal for recovery after SCI (Ying et al. 2008; Weishaupt et al. 2013). The level of BDNF is also elevated after DHA treatment and a synergistic effect with exercise has been demonstrated in different animal models (Wu et al. 2008; Wu et al. 2013). Therefore, some of the functional effects seen in this study might be mediated by an increase in overall activity in motor systems affected by increased availability of BDNF signalling.

Additionally, the synergistic effect of training and DHA might be attributable to a significant neuroprotective effect of DHA at an early stage of SCI. In our group's previous studies, DHA has been shown to provide neuroprotection when administered

acutely, consistent with reducing cell apoptosis and preventing axon demyelination effects. On the other hand, increased neuronal survival after exercise was also reported to be associated with elevated expression of several key intermediates of the PI3K/Akt pathway (Chen et al. 2005). That might imply that functional recovery is a consequence of a synergistic effect on neuroprotection.

7.5 Summary

- On the basis of what is reported in the present study, the hypothesis that a synergistic effect of DHA and rehabilitative training contributes to functional recovery via synaptogenesis and neuroplasticity processes, is strongly supported.
- These data demonstrate that implementing task-specific training shortly after SCI is advantageous up to 3 weeks after injury.
- In our hemisection animal model, the rehabilitation training improved specific-task function; however, there was no negative impact on a non-trained task.
- In the combined treatment group, tissue caudal to the lesion site showed a significant increase in axonal sprouting, and a trend towards increased synaptic contacts, which in combination may promote functional recovery.

8 General discussion

8.1 Summary

The most promising findings from the experiments carried out in this thesis were as follows: 1) Characterization a cervical SCI animal model by applying a left hemisection at C4/5 level. This model is useful to determine the neurological deficit following cervical SCI in the acute stage. A recovery of locomotion was observed in our animal model; however, the ability to exert fine motor control was not regained. 2) The therapeutic effects of an acute DHA injection (250 nmol/kg) following cervical SCI are demonstrated by skilled movement recovery. The significant neurological functional improvement is likely to be related to neuroprotection by DHA. More neuronal cells survived at 3 weeks in the epicentre site after injury. An overall modest effect of DHA was observed on microglial activation and lesion size reduction. 3) Parallel to the neuroprotective effect of DHA, we also demonstrate that DHA promotes neuroplasticity in cervical SCI. The histological analysis showed that DHA treatment can up-regulate synapse formation and promote axonal sprouting of CST and increase serotonin fibres. In an alternative injury model and species, mouse pyramidotomy, acute DHA administration was found to improve forelimb skilled locomotion at a higher dose (500 nmol/kg). In addition to enhanced CST sprouting after treatment, the quantitative analysis also showed that the number of V2a interneurons contacting sprouting fibres was positively related to the functional recovery. 4) In an effort to explore the possible mechanism underlying the neuroplasticity promoting effect, we observed an altered expression of miR21 and PTEN in CST neurons one day after cervical hemisection in an *in vivo* study. In an *in*

vitro study, a primary cell culture of DRG neurons was treated with different concentrations of DHA to demonstrate the neurite promoting growth effect of DHA. Subsequently, a PTEN activator (sodium selenite) was used to determine whether PTEN plays a critical role in promoting axonal growth, and to gain more insight into the mechanism of acutely administered DHA-induced neuroplasticity following traumatic SCI. 5) Finally, I combined the task specific rehabilitation with acute administered DHA treatment to examine the synergistic effect in terms of specific neurological functional recovery. The combined therapy had a significantly beneficial impact on traumatic SCI compared to each single treatment.

Several conclusions have been drawn from this study, but a number of fields should be further explored. Some of these will be summarized and discussed in the following sections.

8.2 Promoting neuroplasticity: a novel effect of DHA following SCI

An extensive body of evidence shows that the neuroprotective effect of DHA can contribute to neurological functional recovery in thoracic SCI animal models. In this thesis, chapter 5 and chapter 6 together provide a novel insight into the mechanism underlying the effect of DHA promotion of neuroplasticity following SCI *in vivo* and *in vitro*, showing that acute DHA administration can enhance axonal fibre sprouting through a mechanism involving miR21 upregulation and PTEN downregulation.

In this thesis, we show that functional recovery is promoted after acute administration of DHA following cervical SCI. However, the question is raised as to whether the functional recovery is just linked to the neuroprotection effect of DHA. In my fourth chapter, the acute delivery of DHA is shown to alleviate the loss of neuronal cells in the epicentre of lesion; the data are consistent with our previous study in the rat thoracic hemisection model (King et al. 2006). Our spinal cord lesion is located between C4 and C5 levels which are relevant to shoulder movement and gross forelimb movement. It is obvious that the acute administration of DHA can significantly improve locomotor functional recovery within one week following cervical hemisection, which could reflect the neuroprotective effect of DHA treatment. However, the skilled forepaw function with fine control is dependent on the interneurons and motoneurons of C6-T1 (McKenna et al. 2000). From the quantitative analysis of immunochemical staining, there seems to be no significant difference between DHA and vehicle group in the number of neuronal cells caudal to the lesion site. Another key feature of DHA treatment that emerged from our analysis is the axonal sprouting that occurs from the spared CST tract and synaptic upregulation. Thus, the modulation of synaptic function and axonal sprouting play a vital part in achieving this complex task.

Recently, numerous agents have been shown to improve functional recovery following SCI. However, it appears that several chemical compounds could possess multifaceted effects on SCI treatment. Systemic administration of some non-steroidal anti-inflammatory drugs have been investigated such as ibuprofen whose neuroprotective effects have been linked with inhibition of inflammation following SCI. However,

ibuprofen recently has been shown to promote locomotion recovery in rats via Rho-A inhibition, in a manner related to a neuroplasticity effect (Fu et al. 2007). On the other hand, ChABC, a promising compound used to promote axonal sprouting or regeneration following CNS injury, was found in one study to ameliorate degenerative changes in the cell bodies of injured CNS projection neurons (Carter et al. 2008). This suggests that neuroprotection may be a possible mechanism of ChABC-mediated repair. Neurotrophin-induced sprouting of descending cortico-spinal tract projections has been shown to depend on immune cell activation (Chen et al. 2008). These examples demonstrate that neuroprotective or neuroplasticity-promoting strategies may be linked to each other by a variety of mechanisms.

Another issue which has to be taken into consideration when evaluating the efficacy of plasticity-promoting treatments is the animal model design. It is an unquestionable fact that the functional recovery related to plasticity promoting strategies depends on a certain degree of spared neuronal tissue being available. In a complete transection animal model, because of no sparing of neuronal tissues following injury, neuroplasticity events that happen above the lesion site will fail to rebuild the circuits below the lesion site which are necessary to promote functional recovery. To access the efficacy of plasticity-promoting treatment, it is essential to understand what degree of injury severity and what kind of injury model and behavioural assessment should be applied. In thoracic SCI animal models, several animal studies suggested that if as little as 10-15% of the spinal cord is spared, stepping abilities and overground locomotion can recover (Schucht et al. 2002). In contrast, in cervical SCI animal models, there appears

to be no recovery in food grasping ability even just in a partial lesion. From previous work in our lab, it is well accepted that DHA has promising neuroprotective effects in rodent contusion or compression models. However, when using models with extensive lesions, like contusion or compression injury, the neuroprotective effects of DHA may play an essential role in functional recovery. Thus we might underestimate or completely mask the potential plasticity-promoting effect of DHA. As a result, here we applied a cervical hemisection spinal cord injury animal model, which has less neuronal damage and correlated well with clinical SCI, to evaluate the plasticity-promoting effect of DHA.

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8.3 The therapeutic effect of acute DHA bolus injection

The cascade of events that occurs following SCI involves a sequence of processes. The vital role of acute DHA administration post SCI has been demonstrated in our group's previous work. Continuous dietary DHA supplementation without an initial acute bolus DHA injection did not confer any neurological functional recovery in rat or mouse SCI (Huang et al. 2007; Lim et al. 2013). Other studies also found that DHA pretreatment has a therapeutic effect and improves neurological outcome following SCI (Figueroa et al. 2012). The lack of efficacy of DHA dietary treatment alone may simply be due to insufficient DHA reaching the spinal cord tissue during the critical time window after SCI, and may indicate that a rapid intervention with intravenous DHA or pretreatment with DHA is essential. However, pretreatment with DHA of patients who are suffering SCI is impracticable clinically. The acute bolus of DHA seems an ideal approach for patients suffering SCI, in the acute stage.

It is also important to note that the combination of acute DHA administration with dietary DHA achieved a better neurologic effect compared to acute DHA treatment alone in rat thoracic SCI (Huang et al. 2007). As we know, the concept of neuroprotection is based on the premise that attenuating the aforementioned pathophysiological process following primary injury will contribute to neurological functional recovery. The therapeutic window for neuroprotective agents is restricted to an early time following injury. Chronic DHA enriched diet could support functional improvement by means of promoting neuroplasticity. It will be essential to analyse in future, the neuroplasticity events in the spinal cord associated with DHA provided chronically in the diet.

8.4 Consideration of the histological evaluation following cervical SCI

The histological finding in our studies showed robust changes related to SCI with/without treatment. Regarding anatomical plasticity, we have demonstrated that behavioural recovery is correlated with sprouting fibres contacting interneurons and motor neurons. However, the conduction properties of individual sprouting fibres following spinal cord injury have not been studied. There is a paucity of information regarding the functional status of these sprouting fibres, whether the sprouting fibres make contact with the appropriate neurons, whether function can be restored because of sprouting fibres, and whether the sprouting fibres are a major contributor to functional recovery. In addition, surviving axons following injury might play a role in functional

improvement. Immunohistological analysis is limited methodologically when there are problem involved the neurological functional evaluation.

In future work, we could perform a detailed functional and anatomical assessment to address this issue by an electrophysiological approach. In the meantime, we can also correlate changes in conduction with quantitative histological data and behavioural analysis, to elucidate the potential neuroplasticity mechanism responsible for functional recovery. Neuroplasticity may also provide the right support for sensory information to guide the formation of useful connections.

8.5 Modulation of PTEN and miR-21 following SCI and DHA treatment

The neuroplasticity-promoting effect of the DHA acute intravenous injection led us to explore one possible underlying mechanism in acute stage. One of the targets of particular interest is the PTEN pathway, which is reported to be a central negative regulator of the PI3K/Akt signalling pathway. This protein, which is present in most central neurons, has been reported to be up-regulated following SCI, with a peak at one day after traumatic injury, and then reduced at 3 days (Ding et al., 2013; Hu et al., 2013). PTEN has been shown to be involved in axonal regeneration in optic nerve (Park et al., 2008), corticospinal neurones (Liu et al., 2010), sensory neurons (Christie et al., 2010) and in synaptic plasticity (Liu et al., 2012). PTEN is also an important target of miR-21. Some studies report that upregulation of miR-21 can improve functional recovery by suppressing PTEN (Han et al., 2014; Sandhir et al., 2014). Our preliminary observations

suggest that the neuroplasticity promoting effect of DHA may be partly associated with its action on miR21 upregulation and PTEN suppression following SCI.

However, we did not reveal the exact casual effect of miR21 and PTEN expression under DHA treatment. Although miR-21 is shown to target negative regulators of PTEN, overexpression of miR-21 also protects against neuronal death, probably mediated by its downregulation of FASLG (Fas ligand (TNF superfamily, member 6)), an important cell death-inducing ligand (Buller et al. 2010). DHA is also known as endogenous ligand for PPARγ. PPARγ agonists also show the ability to suppress PTEN expression and increase Akt phosphorylation in ischaemia-reperfusion cell line (Kim et al. 2011). DHA might inhibit PTEN expression by PPAR-γ mediated pathway. Furthermore, our findings provide a counterbalance to proposed strategies in breast cancer treatment, in which DHA treatment blocks miR-21, thereby increasing PTEN (Mandal et al. 2012). The opposite response of neurons and cancer cells could be related to differences in phosphatidylinositol species of phosphatidylinositol phosphates (PIPs) (Gu et al. 2013). It will be essential in future studies to define the signalling molecules that activate miR-21 and suppress PTEN in response to DHA treatment.

8.6 Neurogenesis effects of DHA following SCI

Adult neurogenesis is limited in mammalian spinal cord. Although the cord retains multipotent neuronal stem cells that could generate functional neurons *in vitro* (Yamamoto et al. 2001; Dromard et al. 2008), production of new neurons by such endogenous cells occurs to only a very limited extent after injury *in vivo* (Yang et al.

2006; Wrathall et al. 2008; Grande et al. 2013). Manipulated differentiation of endogenous stem cells with growth factors and transcription factors could stimulate the production of new neurons and oligodendrocytes (Ohori et al. 2006). Interestingly, a recent study showed that astrocytes, which are highly activated following SCI, can be converted to neuroblasts by a single transcription factor, SOX2, in the injured adult spinal cord. The induced neuroblasts can give rise to synapse-forming interneurons (Su et al. 2014). These results indicated that neurogenesis could be completed by conversion of endogenous cells to neurons following SCI.

DHA has shown promising effects on neurogenesis *in vivo* and *in vitro* (Kawakita et al. 2006; Dyall et al. 2010). Reduced neurogenesis in the cerebral cortex (ventricular and subventricular zones) and dentate gyrus is found in the DHA deficient embryonic rat brain (Coti Bertrand et al. 2006). On the contrary, adult fat-1 transgenic mice with high endogenous DHA levels revealed an increased number of proliferating neurons and neuritogenesis in hippocampus, which correlated with better spatial learning performance (He et al. 2009). However, the underlying mechanism of the neurogenesis promoting effect of DHA has not been fully established. Some studies have revealed that DHA as well as other omega-3 fatty acids broadly regulate the expression of genes (Kitajka et al. 2002; Deckelbaum et al. 2006). The expression of genes could be regulated at the transcriptional level by nuclear receptors. DHA is an endogenous ligand for PPARs, which are nuclear transcription factors and play a vital role in neural stem cell proliferation and differentiation (Wada et al. 2006; Mullen et al. 2007). A recent study showed that a PPAR agonist can promote neural stem cells differentiation by

upregulating SOX2 levels (Bernal et al. 2015), which suggests that DHA might have the potential to promote neurogenesis following SCI by increasing SOX2-induced neurons from astrocytes.

In addition to PPAR activation, the PUFA–GPR40 interaction might have a critical role in neurogenesis in the adult primate hippocampus (Yamashima 2008; Boneva et al. 2011). DHA can also promote the neuronal differentiation of rat neural stem cells via the GPR40-linked signalling pathway (Ma et al. 2010). Furthermore, one study reported that DHA may elicit the GPR40-linked signalling pathway for the regulation of adult bone marrow-derived stromal cells (BMSC), leading to expression of neuronal markers *in vitro* (Kaplamadzhiev et al. 2010). Therefore, it is possible that DHA may act as an extracellular signaling molecule at the membrane of the GPR40 receptor to regulate neurogenesis following SCI.

8.7 Future work

8.7.1 Do we need a different administration route or a higher dose of DHA?

The route of administration of DHA after injury is of interest for future potential clinical applications. Previous studies have reported that after intravenous radiolabelled fatty acid injection, omega-6 PUFAs have a rapid disappearance from plasma with a half-life of less than 1 min, and that only 1% of the injected dose is rapidly incorporated into brain lipid, particularly phospholipids (Robinson et al. 1992; Rapoport 2001; Rapoport 2003). Direct delivery in the CNS could be considered as another effective route of administration of DHA after SCI, such as intrathecal injection or topical application at the

lesion site. It has been suggested that intrathecal injection or intracerebroventricular injection of DHA contributed to neuropathic pain relief in experimental studies (Nakamoto et al. 2012; Lu et al. 2013). Topical DHA treatment also enhanced corneal nerve regeneration after an epithelial wound (Esquenazi et al. 2005). A treatment strategy following incomplete SCI is surgical intervention, which aims to decompress, realign and stabilise the injured cord. Immediate decompression alleviates the impact of secondary injury following SCI. From a neurosurgeon's viewpoint, it would be very interesting to explore the effect of DHA applied directly to the injured spinal cord after surgical intervention.

In our study project, DHA at the dose the 250 nmol/kg delivered to hemisection injury rats is adapted from our group's previous data in rat thoracic SCI animal models (Huang et al. 2007). In this study in rats, no clear dose-dependent effect was seen with the bolus of DHA post-SCI, and no obvious toxicity was observed when DHA 2500 nmol/kg was injected 1 h after thoracic SCI. As discussed in chapter 3, the pathology and functional recovery after cervical SCI are rather different from that observed in thoracic SCI. In thoracic SCI animal models, locomotor evaluation is the main neurological assessment following injury. Subtle neurological recovery due to neuroplastic changes may not be observed in locomotor function following thoracic SCI. More detailed specific skilled and segmental neurological function recovery is easy to identify in the cervical SCI animal model. Therefore, a more extensive examination of higher doses of DHA would be useful in the future. It is essential to keep in mind that the beneficial effects of DHA may follow an inverted U-shaped curve. In a model of cerebral ischaemia, one study reported that a DHA–albumin complex exerts neuroprotective effect at 0.63

mg/kg, but not at 1.25 mg/kg (Belayev et al. 2005). Multiple dose studies in cervical SCI models should be considered in the future to achieve optimal therapeutic effect.

8.7.2 Negative effects of neuroplasticity

Another principle is that not all plasticity has a positive impact on clinical status; in some cases, plasticity might have negative consequences. Maladaptive neuroplasticity in patients following SCI includes muscle spasticity, chronic pain, allodynia, autonomic dysreflexia (Brown et al. 2012). In experimental studies, numerous data have showed that treatment aimed at enhancing spinal plasticity which is incorrectly directed can lead to enhanced pain states and autonomic dysreflexia (Romero et al. 2000; Hofstetter et al. 2005; Weaver et al. 2006). Neuropathic pain following SCI has many underlying factors such as an increase in neuronal excitability due to the products of microglial activation, changes in sodium channel expression and changes in glutamate receptor expression (Deumens et al. 2008). The increased density of serotonergic axons rostral to the SCI in one study appeared to promote at-level neuropathic pain through actions of serotonin on pro-nociceptive 5-HT_3 receptors (Oatway et al. 2004). Another report revealed that the neuropathic pain is related to the collateral sprouting of calcitonin gene-related peptide (CGRP) containing primary afferent fibres in the spinal cord dorsal horn (Christensen et al. 1997). Strategies to promote neural plasticity also have possible effects on the growth of CGRP expressing fibres in the injured spinal cord and may affect neurological outcome. Such strategies include manipulation of NGF expression (Cameron et al. 2006), or OEG transplantation (Richter et al. 2005). In future studies, we could assess mechanical sensitivity after SCI by measuring the paw withdrawal threshold using Von Frey test (Chaplan et al. 1994) and thermal sensitivity by

measuring the time taken for a radiant heat source to elicit a flexion reflex, using Hargreaves' method (Hargreaves et al. 1988).

8.7.3 Are there are any other mechanisms contributing to the neuroplasticity promoting effect of DHA?

DHA is an endogenous ligand for RXR, which is one of several members of the retinoic acid nuclear receptor family. After SCI, RXRs appear in the cell nuclei of reactive microglia, macrophages and neuronal cells. This RXRs expression began at 4 days, was most prominent at 7 and 14 days and had decreased at 21 days after injury (Schrage et al. 2006). It is possible that the DHA bolus could target transcription factors and influence retinoic acid signalling after SCI. Several studies have demonstrated that retinoid pathway activation can promote neurite outgrowth *in vitro* (Corcoran et al. 2000) and axonal regeneration *in vivo* (Taha et al. 2004). In a SCI experimental study, a retinoic acid receptor β (RARβ) agonist was shown to act through PI3K signalling to induce axonal outgrowth of CST fibres and promote functional recovery (Agudo et al. 2010). Fenretinide, a semisynthetic analogue of retinoic acid, also reduces tissue damage and promotes neurological functional recovery following SCI (Lopez-Vales et al. 2010). Of note, the recovery of neurological function is accompanied by reduced AA but increased DHA levels in plasma and injured spinal cord tissue. DHA is also an endogenous ligand of PPARs. One study showed that PPARγ is crucial for coupling ibuprofen to RhoA inhibition and a subsequent neurite growth in neurons. RhoA inactivation with PPARγ agonists overcomes the growth restriction of CNS axon inhibitors (Dill et al. 2010). Therefore, it will be interesting to determine if the effect of

DHA on promoting neuroplasticity is linked to the retinoid pathway or PPARs activated by the bolus DHA administration following SCI. In fact, dissection of this molecular pathway may offer novel molecular targets for effective SCI treatment.

Apart from promoting intrinsic regrowth ability, DHA appears to have the ability to modify the glial scar after SCI (our group unpublished data). Glial scar formation is a reactive cellular process involving astrogliosis that inhibits axonal growth following SCI. One strategy to promote axonal growth is to overcome the inhibitory environment. A recent study has demonstrated a potential role for miR-21 in regulating astrogliosis after SCI by using transgenic mice that overexpress in astrocytes either miR-21 or a miRNA sponge designed to inhibit miR-21 function (Bhalala et al. 2012). The result indicated that manipulation of miR-21 can modulate the astrocyte expression in the SCI chronic phase. Further studies could focus on whether the glial scar modified by DHA treatment correlated with axonal sprouting or neurological functional recovery in SCI models.

8.7.4 Are other omega-3/omega-6 PUFAs beneficial to neuroplasticity after SCI?

In addition to DHA, previous data from our group showed that EPA and AA also have the ability to enhance neurite growth in DRG cell cultures (Robson et al. 2010). Dietary supplementation with EPA leads to a significant increase in synaptic proteins such as synapsin-1, postsynaptic density-95 protein and syntaxin 3 in brain (Cansev et al. 2007). It is therefore worth investigating further the other omega-3/omega-6 PUFAs, that might have an effect on neuroplasticity or synaptogenesis in SCI.

To summarize, DHA has emerged as a substance with significant therapeutic potential in various neurological diseases. This thesis has assessed the neuroplasticity promoting effect of DHA *in vivo* and *in vitro* following SCI, which shows much promise as a potential therapy for the treatment of SCI.

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Appendices

Table 1 Basso, Beattie, and Bresnahan (BBB) locomotor rating scale used to assess hindlimb function after rats SCI (Basso et al., 1996)

Score	
0	No observable hindlimb (HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support or plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps; no FL–HL coordination
11	Frequent to consistent weight-supported plantar steps and no FL–HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL–HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL–HL coordination
14	Consistent weight-supported plantar steps, consistent FL–HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL–HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off
18	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent FL–HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift off, and tail is down part or all of the time
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

Table 2 Forelimb Locomotor Scale (FLS) used to assess forelimb function after rats SCI (Cao et al., 2008)

Score	
0	No movements of the forelimb (shoulder, elbow or wrist joints)
1	Slight movements of one or two joints of the forelimb
2	Extensive movement of one joint and slight movement of another joint of the forelimb
3	Slight movement of all three joints of the forelimb
4	Extensive movement of one joint and slight movement of two joints of the forelimb
5	Extensive movement of two joints and slight movement of one joint of the forelimb
6	Extensive movement of all three joints of the forelimb
7	Plantar placement of the forelimb with no weight support
8	Dorsal stepping only
9	Dorsal stepping and/or occasional plantar stepping
10	Frequent plantar stepping
11	Continuous plantar stepping
12	Continuous plantar stepping with paw position rotated (either at initial contact, lift off or both)
13	Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both)
14	Continuous plantar stepping with paw position rotated (either at initial contact, lift off or both) and occasional toe clearance
15	Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both) and occasional toe clearance
16	Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both) and frequent toe clearance
17	Continuous plantar stepping with paw position parallel (either at initial contact, lift off or both) and continuous toe clearance

Figure 1. The correlation between histological finding and behavioural recovery